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PRINCIPAL INVESTIGATOR: Amy Brooks-Kayal, MD

CONTRACTING ORGANIZATION: University of Colorado Denver
Aurora, CO 80045

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6. AUTHOR(S)
Dr. Amy Brooks-Kayal, MD, Lauren Frey, MD and Bret N. Smith, PhD

E-Mail: amy.brooks-kayal@childrenscolorado.org

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14. ABSTRACT
Traumatic Brain Injury (TBI) is a well-established inducer of temporal lobe epilepsy (TLE), a frequently medically intractable epilepsy syndrome. The controlled cortical impact (CCI) model of posttraumatic epilepsy in mice is a well established animal model of TBI that results in localized cell loss, synaptic reorganization, and development of TLE. Abnormalities in inhibitory neurotransmission are important aspects of TLE in several animal models. Under this award, the CCI model was established in the two collaborating universities. Specific parameters of injury associated with epileptogenesis were determined. It was determined that upregulation of the JaK/STAT pathway in the injured hippocampus occurs after CCI and was associated with changes in GABA(A) receptor (GABAR) subunit changes, which could be blocked by post-injury administration of a JaK/STAT inhibitor, WP1066. Blocking JaK/STAT3 activity did not prevent loss of GABA cells in the injured hippocampus. Inhibitory postsynaptic currents in the dentate gyrus ipsilateral to the injury were reduced in frequency weeks after the injury. Post-injury administration of a JaK/STAT3 inhibitor did not reduce development of post-traumatic epilepsy, and did not significantly improve memory function, but did enhance the motor recovery. These findings support a role for the JaK/STAT pathway in GABAR regulation and suggest the potential of JAK/STAT inhibitors to enhance recovery after TBI.

15. SUBJECT TERMS
dentate gyrus, epileptogenesis, GABA(A) receptor, JAK/STAT, posttraumatic epilepsy

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Table of Contents

Progress Report Summary #W81XWH-11-1-0501 (July 2012-June 2013)

INTRODUCTION:

This research addresses the FY10 PRMRP topic area of Epilepsy. Traumatic Brain Injury (TBI) is a well-established etiology of temporal lobe epilepsy (TLE), a frequently medically intractable and often progressive epilepsy syndrome. Much evidence indicates that abnormalities in inhibitory neurotransmission are important in TLE. Our overall hypothesis is that Janus Kinase (JaK)/Signal Transducer and Activator of Transcription 3 (STAT3) pathway activation after TBI leads to GABA(A) receptor α1 subunit gene (*Gabra1*) repression and is a critical mediator of post-traumatic epileptogenesis and epilepsy progression. The JaK/STAT pathway has not been studied in post-traumatic epilepsy, but beyond its role in *Gabra1* regulation, it is known to be an important regulator of neuronal proliferation, survival and gliogenesis, all of which may be important contributors to epileptogenesis. Specifically, long-term decreases in expression of the GABA(A) Receptor α1 subunit gene (*Gabra1)* in the hippocampal dentate gyrus have been shown to occur in the pilocarpine-model of TLE in animals and preventing this repression has been shown to inhibit development of epilepsy in this model. In addition, decreases in expression of α4 subunits, which contribute to the generation of tonic GABA currents and potently regulate granule cell activity, have also been reported in TLE and after TBI. It has been recently established that transcriptional repression of GABA receptor subunits in the pilocarpine-model of TLE is mediated by inducible cAMP early repressor and phosphorylated CREB, and that ICER transcription is driven by the JaK/STAT signaling cascade. Pharmacological inhibition of the JaK/STAT3 pathway prevents *Gabra1* repression and inhibits progression of epilepsy in the pilocarpine model. Preliminary data indicated that spontaneous seizures activate the JaK/STAT3 pathway in the pilocarpine model, suggesting this pathway may be involved in the maintenance and progression of TLE. Preliminary evidence shows that the JaK/STAT3 pathway is activated following TBI in injured hippocampus and cortex after a *diffuse* injury, and data obtained under this award indicate that phosphorylated STAT3 is increased shortly after focal injury near the injury site, with concurrent changes in α1 and α4 GABA receptor subunit expression. The impact however, on GABA(A) receptor (GABAR) subunit function, and whether these are important mechanisms of post-traumatic epileptogenesis are unknown. Focal injury caused by controlled cortical impact (CCI) has been shown to induce cell loss, synaptic reorganization, and TLE with spontaneous seizures in mice (Hunt et al., 2009; 2012).

The Specific Aims of our study were:

1. Determine whether activation of the JaK/STAT pathway and downregulation of GABA(A) Receptor α1 subunit gene (*Gabra1*) transcription occur following traumatic brain injury (TBI) and subsequent epileptogenesis. We hypothesized that activation of the JaK/STAT pathway, downregulation of Gabra1 and subsequent reduction of **α**1 subunit levels occur in injured hippocampus and cortex following TBI and contribute to posttraumatic epileptogenesis. To test this hypothesis, we examined activation of the JaK/STAT pathway, and levels of ICER and GABAR subunits acutely and chronically in the controlled cortical impact (CCI) model in mice before and after development of spontaneous seizures.

2. Determine whether activation of the JaK/STAT pathway and downregulation of *Gabra1* transcription following TBI result in altered inhibitory synaptic neurotransmission in the hippocampus that may contribute to epileptogenesis. We hypothesized that after CCI inhibitory neurotransmission in the dentate gyrus would be altered in a fashion consistent with reduced GABAR ***α***1 subunit surface expression in association with JaK/STAT pathway activation and *Gabra1* downregulation. To test this hypothesis, we examined GABAR-mediated currents in dentate gyrus granule cells using whole-cell patch-clamp recordings in acute hippocampal slices from injured and uninjured mice.

3. Determine whether animals can be rescued from post-traumatic epilepsy development and/or progression via blockade of JaK/STAT pathway activation acutely after controlled cortical impact or chronically after onset of spontaneous seizures. We hypothesized that blockade of the JaK/STAT pathway would inhibit epilepsy development and/or progression after CCI. To address this hypothesis, we pharmacologically inhibited STAT3 phosphorylation after CCI, then used video electroencephalogram (EEG) monitoring to determine whether this treatment prevents or delays epilepsy development and/or progression and also examined motor and memory recovery.

Results of these studies provided new information regarding the role of the JaK/STAT signaling cascade in regulation of brain inhibition and epileptogenesis after traumatic brain injury, and have supported the promise of JaK/STAT inhibitors as potential therapies to enhance motor recovery after TBI.

BODY:

Aim I: Performed in laboratory of Dr. Amy Brooks-Kayal at University of Colorado

Task 1: Determine whether activation of the JaK/STAT pathway occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12)

1a. Induce TBI using the CCI model in adult CD-1 mice (200 mice- assuming that 25-30% loss due to death or suboptimal injury; Time frame months 1-10).

Status: Completed. CCI of either "moderate" and "severe" severity has been completed on approximately 400 mice.

The CCI model was not in use at UCD prior to the start of this project and purchase, setup and training was essential for the completion of all subsequent studies.

1b. Sacrifice mice at 6, 24, 48, 72 hrs, 7 days and 16 weeks after CCI (150 mice; Timeframe months 1-4).

Status: Completed. Injured mice have been sacrificed at 6 hours, 24 hours, 48 hours, 72 hours, 1 week and 10-16 weeks after injury.

Background necessary for this subtask has also been completed and includes:

1. Training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains

Accomplishments: Harvested tissue was used for studies of STAT3 phosphorylation at all above time points (see **1c and 1d** below).

1c. Measure levels of mRNA for JaK1 and 2, STAT1-5 in microdissected subregions of hippocampus (DG, CA1, CA3) and in cortex ipsilateral and contralateral to injury using quantitative reverse transcription polymerase chain reaction (qRT-PCR) (50 mice; Timeframe months 4-7).

Status: Not completed

The following background work was completed for this subtask:

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Tested mRNA protocols for measuring JaK1 and 2 and pSTAT1-5 using quantitative reverse transcription polymerase chain reaction (qRT-PCR)

However, the mRNA protocols, as designed, were ultimately technically limited and the data generated from these protocols was of limited reliability.

Accomplishments: Not completed

Supporting Data: None

1d. Measure protein levels of JaK1 and 2, pSTAT1-5 levels using western blotting in homogenates of microdissected brain regions (50 mice; Timeframe months 6-9). Status: Completed

1. Completed training of study personnel in non-traumatic dissection techniques to obtain cortical and hippocampal tissue samples from mouse brains
2. Optimized and implemented Western blot protocols for protein measurements of JaK1 and 2 and pSTAT1-5 in mouse brain

Based on this work and other work done in the lab, pSTAT3 has been the most reliable indicator of JaK/STAT pathway activation in multiple models of rodent epileptogenesis and, as such, has formed the focus of this investigation. CCI of both moderate (1mm, 3.5 m/s, 400ms) and severe (2mm, 5m/s, 199ms) severity was used for this task (Figure 1). Whole hippocampus was dissected from injured and sham mice at 6 hours, 24 hours, 48 hours, 72 hours, 1 week and 12-16 weeks after CCI. Western blots of protein homogenates from whole hippocampus were probed with pJaK2 (24 hour timepoint), JaK2 (24 hour timepoint), pSTAT3 (all timepoints) and STAT3 (all timepoints) antibodies. Quantification of western

blot analysis was performed and pSTAT3 and pJaK2 levels were normalized to STAT3 or JaK2 levels respectively and expressed as a % change compared to shams.

Accomplishments: These studies demonstrated that pSTAT3 are significantly increased in injured mouse hippocampus after CCI of both moderate and severe severity. In addition, pSTAT3 levels are significantly greater in the more severely injured animals.
These results established that there is an increase in activation of STAT3 following CCI in mice, similar in magnitude and time course to that seen in rat (see related studies, ***Raible et al., J Neurotrauma, 2012 Sep 4. [Epub ahead of print], PMID: 22827467****. Appendix item #1), and suggest that the JaK/STAT pathway may be differentially activated based on injury severity.*

Supporting Data: **Figures 1 and 2**.

1e. Assess protein levels and regional/cellular expression of JaK1 and 2, pSTAT1-5 using fluorescent immunohistochemistry with co-staining for cell specific markers (50 mice; Timeframe months 9-12).
Status: Not completed
Accomplishments: Not completed
Supporting Data: None

Task 2: Determine whether upregulation of *ICER* transcription occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12: studies will overlap with Task 1 and be performed in same animals)
2a. Induce TBI using the CCI model in adult CD-1 mice (200 mice [same mice used in Task 1a]; months 1-10)
Status: Completed. See **1a** above.

2b. Sacrifice mice at 6 and 24 hrs, 7 days and 10 weeks after CCI (150 mice [same mice used in Task 1b]; months 1-4)
Status: Completed. See 1b above.

2c. Measure levels of mRNA for ICER in brain regions ipsilateral and contralateral to injury using qRT-PCR (50 mice [same 50 mice as used in Task 1c]; months 4-7).
Status: Completed
Accomplishments: ICER mRNA levels shown to increase ~5-fold after FPI
Supporting Data: None (see Raible et al 2012)

2d. Measure protein levels of ICER using western blotting in brain regions (50 mice [same 50 mice as used in Task 1d]; Timeframe months 6-9).
Status: Not completed
Accomplishments: Not completed
Supporting Data: None

Studies similar to Task 2 have been completed in a related rodent model of posttraumatic epileptogenesis (fluid percussion injury). Poor antibody specificity limited the value of the results of these related studies so studies for Tasks 2d- 2e were not completed.

2e. Assess protein levels and regional/cellular expression of ICER using fluorescent immunohistochemistry (50 mice [same 50 mice as used in Task 1e]; Timeframe months 9-12).
Status: Not completed

Accomplishments: Not completed
Supporting Data: None

Task 3: Determine whether downregulation of *Gabra1* transcription occurs following traumatic brain injury (TBI) and subsequent epileptogenesis. (Timeframe months 1-12: studies will overlap with Tasks 1 and 2 and be performed in same animals)
3a. Induce TBI using the CCI model in adult CD-1 mice (200 mice- assuming that 25- 30% loss due to death or suboptimal injury; months 1-10)
Status: Completed. See **1a** above.

3b. Sacrifice mice at 6 and 24 hrs, 7 days and 10 weeks after CCI (150 mice [same used in Task 1b]; months 1-4)
Status: Completed. See 1b above.
Accomplishments: Harvested tissue was used for studies of *Gabr* subunit protein and mRNA levels (see **3c and 3d** below).

3c. Measure levels of mRNA for *Gabr* subunits in brain regions ipsilateral and contralateral to injury using qRT-PCR (50 mice [same mice as used in Task 1c]; months 4-7).
Status: Completed
Accomplishments: α1 expression is decreased in both CCI-S and CCI-M injured mice 6 hours, 24 hours, and 48 hours after CCI.
Supporting Data: **Figure 3**

3d. Measure protein levels of *Gabr* subunit levels using western blotting in brain regions (50 mice [same mice used in Task 1d]; months 6-9).
Status: Completed

Western blots were performed using whole hippocampal protein collected from mice 12, 14, 16 weeks after CCI and probed with anti-GABAAR α1, α4, γ2, δ, α2, α5, β2, β3 and β-actin antibodies. $GABA_AR$ subunit levels were normalized to β-actin levels and expressed as a percent change compared to sham-injured animals.

Accomplishments: Demonstrated that $GABA_AR$ subunit protein level α1 is significantly decreased at 12, 14 and 16 weeks after severe CCI. Also, the $GABA_AR$ subunits α2, α5, β2, and β3 protein levels are not significantly changed in injured hippocampus 1 and 16 weeks after severe CCI or moderate CCI in mouse.
Supporting Data: **Figure 4, Figure 5**

3e. Assess protein levels and regional/cellular expression of *Gabr* subunits using fluorescent immunohistochemistry (50 mice [same mice used in Task 1e]; months 9-12).
Status: Not completed
Accomplishments: Not completed
Supporting Data: None
The fragility of hippocampal tissue following severe CCI injury did not produce thin sectioning required for adequate immunohistochemical analysis so these studies were not completed.

Aim 2: Performed in laboratory of Dr. Bret N. Smith at University of Kentucky

Determine whether activation of the JaK/STAT pathway and downregulation of *Gabra1* transcription following TBI result in altered inhibitory synaptic neurotransmission in the hippocampus that may contribute to epileptogenesis.

Task 1: Determine whether benzodiazepine modulation of IPSCs in dentate granule cells (DGCs) is altered after CCI and whether this alteration is prevented by inhibiting STAT3 phosphorylation with WP1066. (Timeframe months 1-18).

Task 1a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 1-18.

Status: Complete

1. Verified parameters of CCI injury that result in epileptogenesis and markers of the development of epileptic phenotype after 8-12 weeks post-injury. Approximately 50 mice were injured with CCI in the last year, 20 of which were injected with WP-1066. Based on collaborators' findings that more severe injury results in greater STAT3 phosphorylation, studies included a few mice with greater injury.

a. The spatial extent of moderate and severe injury-related epileptogenic changes with respect to distance from impact point was determined.

2. Establish that phosphorylation of STAT3 is upregulated and was inhibited by WP1066 in this model.

a. Establish effect and localization of STAT3 phosphorylation after CCI.
b. Establish effect of WP1066 on STAT3 phosphorylation after CCI.
c. Based on collaborators preliminary results, initiated studies to examine the extent of STAT3 phosphorylation after more severe injury.

Accomplishments:

1. Determined precise parameters of effective CCI (1 mm depth) to obtain epileptogenic phenotype. Phenotype changes were assessed for more severe injury (2 mm), based on preliminary findings from collaborators. In our hands, 2 mm injury essentially destroyed the hippocampus and dentate gyrus and was determined to be too severe to study effects of injury ipsilaterally. With intermediate injury (1.5, 1.2 mm), damage was either similar to 2 mm (i.e. 1.5 mm) or to 1 mm (i.e., 1.2 mm). The 1 mm injury was therefore used for all studies.
2. Completed determination that CCI increased STAT3 phosphorylation ipsilateral to the injury after 24 hours, but not contralaterally. Western blot analyses of hippocampi from CCI-injured and control mice were concluded after 1 mm depth injury. Both STAT and phosphorylated STAT (pSTAT) protein expression were compared semi-quantitatively 24 hr after injury **(Fig 1)**. Comparisons were made for hippocampi ipsilateral to the injury, contralateral to the injury, and in sham-operated controls. STAT and pSTAT levels were normalized to those for β-actin. Results indicated that pSTAT ($p<0.05$), but not STAT ($p>0.05$), expression was increased in the hippocampus ipsilateral to the injury. We further determined that treatment at 30 and 90 min after CCI (1 mm) with WP1066 (50 mg/kg) inhibits STAT3 phosphorylation in mice. Full analysis was reported (Butler et al., 2012; Boychuk et al., 2012). Conclusion: The biochemical reaction required to perform further analyses of GABA currents is evident after CCI ipsilateral to the 1 mm injury, but not contralaterally. This means that the contralateral dentate gyrus can serve as a control for electrophysiological analyses.
3. Completed analysis of histopathological features (i.e., MFS and hilar GABA neuron loss) in the dentate gyrus consistent with epileptogenesis **(Figs. 2, 6)**. Mossy fiber sprouting analysis was

completed in year one (Hunt et al., 2012). Assessment of hilar inhibitory neuron loss was completed in year 2; preliminary data were included in the previous progress report (Butler et al., 2012; Boychuk et al., 2012). The distribution of inhibitory neuron loss was compared to previous results on mossy fiber sprouting. Significant GABA neuron loss was limited to the injury epicenter and an area extending 800 μm temporal (ventral) to the injury; areas septal (dorsal) to the injury were unaffected, similar to the distribution of mossy fiber sprouting. Contralaterally and at more ventral levels ipsilaterally, hippocampal pathology was not observed (Butler et al., 2012). We further determined that treatment with WP1066 did not affect GABA neuron loss. Full analysis of mice from the two injection paradigm (50 mg/kg each), delivered at 30 and 90 min post-injury (1 mm) was completed. The WP1066 treatment did not, however, alter the degree of GABAergic hilar interneuron cell loss ipsilateral to the injury (Butler et al., 2012). Conclusions: The histopathology most likely to be required for studying changes in GABA currents after CCI was associated with hippocampal evulsion, which was observed when using beveled tips (versus rounded tips) to a depth of 1 mm. Treatment with WP1066 showed no evidence of influence on cell proliferation, axon growth, or neuronal survival.

Supporting Data: **Figure 6.** Western blot results indicating increased STAT3 phosphorylation after 1 mm CCI ipsilateral to the injury and inhibition of pSTAT3 by systemic WP1066 treatment (50 mg/kg, i.p.) at 30 and 90 min after CCI injury. **Figure 7.** The hilar GABA neuron loss after CCI and the lack of effect of WP1066 treatment on hilar neuron loss. These data were completed in year 2, but reported in revised progress report for year 1.

Task 1b. Measure effects of zolpidem on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice shortly (i.e., 1-6 weeks) after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 1a; Timeframe months 1-9).

Status: Completed

Accomplishments:

1. IPSCs were recorded from granule cells from sham control, CCI-injured, and CCI-injured+WP1066 treatment mice (1-6 weeks post-injury; 8-12 mice per group). IPSC frequency, amplitude, and decay time constants were measured. Analysis is complete for amplitude and frequency; analysis is being verified for decay time constant and that process will be complete within 30 days.
2. Applied α1 subunit-selective benzodiazepine agonist, zolpidem, and measured IPSC frequency, amplitude and decay time constant in neurons from sham control, CCI-injured, and CCI-injured+WP1066 treatment mice. Analysis is complete for effect of zolpidem on amplitude and frequency; analysis is being verified for effect on decay time constant and that process will be complete within 30 days: benzodiazepine agonist tends to increase IPSC frequency and time constant, and these effects were not statistically different in WP1066-treated mice.

Supporting Data: **Figure 8**. Graphical description of results from IPSC analysis within 10-21 days after injury.

Task 1c. Measure effects of zolpidem on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 1a; Timeframe months 4-18)

Status: Completed

Accomplishments:

1. IPSCs were recorded from granule cells from sham control, CCI-injured, and CCI-injured+WP1066 treatment mice (1-6 weeks post-injury; 8-11 mice per group). IPSC frequency, amplitude, and decay time constants were measured: benzodiazepine agonist tends to increase IPSC frequency and time constant, and these effects were not statistically different in WP1066-treated mice.
2. Applied α1-subunit selective benzodiazepine agonist and measured IPSC frequency, amplitude and decay time constant in neurons from sham control, CCI-injured, and CCI-injured+WP1066 treatment mice. Analysis is complete for effect of zolpidem on amplitude, frequency, and decay time constant: benzodiazepine agonist tends to increase IPSC frequency and time constant, and these effects were not statistically different in WP1066-treated mice.
3. Tonic GABA currents were minimally affected by zolpidem in neurons from control mice. However, at both 1-6 weeks and 10-13 weeks, zolpidem induced a significantly larger tonic current in neurons from CCI-injured mice ($p<0.05$). In neurons from CCI-injured mice treated with WP1066, zolpidem application resulted in a small Itonic that was similar to that observed in control mice and significantly less than that observed in untreated, CCI-injured mice. This effect was observed at both the 1-6 week and 10-18 week timepoints.

Supporting Data: **Figure 9.** Graphical description of results from IPSC analysis 6-10 weeks after injury. **Figure 10.** Graphical depiction of effects of zolpidem on Itonic at early and late time points. See also **Table 1** below for analysis of IPSC amplitude, frequency, and time constant for control, CCI-injured, and CCI-injured+WP1066 treated mice.

Task 1d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 1a-c; Timeframe months 1-18).

Status: Completed

Accomplishments:
1. Approximately 100 mice were treated with CCI, 50 of which were treated with WP1066; 80 controls were used.
2. Timm staining did not revealed mossy fiber sprouting in any group at the early time-point, as expected.
3. At 6-10 weeks post-injury, Timm staining was observed in CCI-injured, but not sham control mice. CCI-injured+WP1066 treated mice displayed Timm distribution similar to CCI-injured mice at 6-10 weeks post-injury.

Supporting Data: See **Figure 11.** Timm scores for the three groups analyzed: Control= $0.13 \text{ x} \pm 0.09$ ($n=16$); CCI-injured= 1.93 ± 0.30 ($n=16$; $p<0.05$ vs control); CCI-injured+WP1066= 1.88 ± 0.30; ($n=16$; $p<0.05$ versus control; $p>0.05$ versus CCI-injured; ANOVA). Mossy fiber sprouting was not reduced by WP1066 treatment after CCI injury.

Task 2: Determine if furosemide modulation of IPSCs in DGCs is altered after CCI and if inhibiting STAT3 phosphorylation with WP1066 prevents the alteration. (Timeframe: months 19-36)

Task 2a. Induce TBI using CCI model in adult CD-1 mice (200 mice used, 20 sham-injured controls, 80 injured untreated, 20 sham-injured, WP1066-treated controls, 80 injured WP1066-treated; Timeframe months 19-36).

Status: Completed

Accomplishments: Accomplishments identical to Task 1, 1a.

Supporting Data: Same as Task 1, 1a.

Task 2b. Measure effects of furosemide on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 1-6 weeks after CCI injury. (100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 2a; Timeframe months 19-27).

Status: Completed.

Accomplishments: Recording experiments were performed in DGCs from 7 mice in each group. Furosemide application decreased IPSC frequency, but not amplitude, in CCI-injured mice relative to control. However, it was determined that furosemide also had non-specific effects on regulators of ionic currents in addition to those mediated by α4-subunit containing GABA receptors, including energy-dependent Cl^- co-transporters. These results were therefore inconclusive.
Supporting Data: none

Task 2c. Measure effects of furosemide on IPSCs in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury (months 4-18). 100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 2a; Timeframe months 19-36).

Status: Completed

Accomplishments: Recording experiments were performed in DGCs from 5 mice in each group. Furosemide application decreased IPSC frequency, but not amplitude, in CCI-injured mice relative to control. However, it was determined that furosemide also had non-specific effects on several currents in addition to those mediated by α4-subunit containing GABA receptors, including energy-dependent Cl^- co-transporters. Results from these experiments were therefore inconclusive.
Supporting Data: none.

Task 2d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 2a-c; Timeframe months 19-36).

Status: Completed

Accomplishments: Accomplishments identical to Task 1, 1d.
Supporting Data: Same as Task 1, 1d.

Task 3: Determine if THIP-induced tonic GABA currents in DGCs are altered after CCI and if the alteration is prevented by inhibiting STAT3 phosphorylation with WP1066. (Timeframe months 10-27)

Task 3a. Induce TBI using CCI model in adult CD-1 mice (100 mice needed, 30 sham-injured controls, 30 injured untreated, 10 sham-injured, WP1066-treated controls, 30 injured WP1066-treated; Timeframe months 10-27)

Status: Completed

Accomplishments: Accomplishments identical to Task 1, 1a.

Supporting Data: Same as Task 1, 1a.

Task 3b. Measure THIP-induced tonic GABA current in DGCs from WP1066-treated and untreated control mice and in mice 1-6 weeks after CCI injury. (100 mice needed, 30 sham-injured controls, 30 injured untreated, 10 sham-injured, WP1066-treated controls, 30 injured WP1066-treated; subset of mice in Task 3a; Timeframe months 10-18).

Status: Completed

Accomplishments: 4,5,6,7-Tetrahydroisoxazolo[5,4*c*]pyridine-3-ol hydrochloride (THIP) currents were measured in neurons from control, CCI-treated, and CCI-injured+WP1066 treatment mice(7-11 mice per group) at 1-6 weeks post injury.

Supporting Data: **Figure 12.** THIP-induced tonic GABA currents are reduced in neurons from CCI-injured mice; WP1066 treatment did not affect the THIP-induced current amplitude at 1-6 weeks post-injury. **Table 1.** THIP application decreased IPSC frequency in all groups to a similar extent. The effect was similar for animals examined at 1-6 weeks and 10-18 weeks. WP1066 had no effect on the THIP effect on IPSCs.

Task 3c. Measure THIP-induced tonic GABA current in DGCs from WP1066-treated and untreated control mice and in mice 6-10 weeks after CCI injury (months 4-18). 100 mice needed, 10 sham-injured controls, 40 injured untreated, 10 sham-injured, WP1066-treated controls, 40 injured WP1066-treated; subset of mice in Task 3a; Timeframe months 19-27).

Status: Completed

Accomplishments:

1. Determined that THIP-induced tonic GABA current in DGCs 6-10 weeks post-injury are reduced in amplitude relative to sham-operated controls and contralateral DGCs (n=7-14 cells in 7 mice from each group; $p<0.05$). Based on preliminary results from collaborators in Colorado and on data published recently elsewhere, these experiments were initiated to identify potential functional changes due to altered α1 vs α 4/δ subunit-containing GABA receptor expression weeks after injury, corresponding to time points where epilepsy is established in this model (i.e., 6-10 weeks post-injury). Granule cells were recorded in ex vivo slices taken from control mice and from slices taken contralateral and ipsilateral to injury site in CCI-injured mice, 6-10 weeks post-injury. Cells were voltage-clamped at 0 mV and THIP (3μM) was bath-applied to induce an outward current, ostensibly due to activation of δ subunit-containing GABA receptors (most likely α4/δ). Bicuculline methiodide (30 μ M) was applied to block all GABA receptors and determine the total available tonic GABA current. Conclusions: Total tonic GABA current in granule cells from control versus CCI-injured mice. THIP-current in cells contralateral to the injury are not different from those in control mice. However, THIP-induced tonic current is reduced by about 50% ipsilateral to the injury in cells from CCI-treated versus controls ($p<0.05$) or in cells contralateral ($p<0.05$) to the injury.
2. Recordings were made from 12 granule cells in 7 WP1066-treated CCI-injured animals and tonic GABA and THIP currents were compared to results from control mice and CCI-injured mice. As in other groups, tonic GABA current was unaffected in WP1066-treated CCI-injured mice ($p>0.05$), as expected. The THIP-current was significantly reduced by about 50% ipsilateral to the injury in cells from WP1066+CCI-treated versus controls or in cells contralateral to the injury ($p<0.05$). The THIP current in the WP1066+CCI-injured group was not different from CCI-treated group ($p>0.05$).

Supporting Data: **Figure 12.** THIP-induced changes in tonic GABA current in sham-operated controls, CCI-injured, and CCI-injured+WP1066 after 6-10 weeks post injury.

Task 3d. Perform Timm histological analysis, to detect mossy fiber sprouting in all slices from which recordings are made. (200 mice needed; same mice as in Tasks 3a-c; Timeframe months 10-27).

Status: in progress

Accomplishments: Accomplishments identical to Task 1, 1d.

Supporting Data: Same as Task 1, 1d.

Aim 3: Performed in laboratory of Dr. Amy Brooks-Kayal at University of Colorado

Task 1: Determine whether inhibition of STAT3 phosphorylation after CCI using WP1066 inhibits post-traumatic epilepsy development (PTE) (Timeframe months 12-21)

1a. Induce TBI using the CCI model in adult CD-1 mice and administer WP1066 or vehicle immediately after injury (200 mice- assuming that 30% loss due to death, suboptimal injury, or loss of implanted recording electrodes; months 12-18)

Status: Completed. CCI has been completed on approximately 400 mice.

1b. Sacrifice mice at 6 hrs, 24 hrs and 7 days after CCI to assess molecular and histochemical effects of WP1066 (120 mice [assuming that 25-30% loss due to death, suboptimal injury]; months 12-15)

Status: Completed

Accomplishments:

1. We performed multiple experiments to identify a dosing paradigm for WP1066 that resulted in inhibition of STAT3 phosphorylation following CCI in mouse, including:

a. First we administered WP1066 50 mg/kg ip either 15 min before or 5 min after CCI injury and harvested hippocampal tissue at 6 hrs after CCI. Western blots of protein homogenates from whole hippocampus were then probed with anti-pSTAT3 and STAT3 antibodies. Quantification of Western blot analysis was performed and pSTAT3 levels were normalized to STAT3 levels and expressed as a % change compared to shams. These studies demonstrated that there was no significant difference in pSTAT3 levels at 6 hrs following CCI in injured hippocampus between vehicle treated and WP1066 treated groups (**Figure 13).**

b. We next examined if 15 minute pre-treatment with a WP1066 analogue with enhanced stability (WP117) at varying doses would inhibit phosphorylation of STAT3 in injured hippocampus 1 hour after CCI. Western blots of protein homogenates from whole hippocampus were performed as described above. These studies demonstrated that there was no significant difference in pSTAT3 levels at 1hr following CCI in injured hippocampus between vehicle treated and WP117 treated groups, and suggested that very early treatment with WP1066 or analogue did not inhibit STAT3 phosphorylation (see **Figure 14**).

c. Next, we administered WP1066 50 mg/kg ip 30 and 90 minute post-CCI and harvested tissue at multiple timepoints after CCI. These studies demonstrated that WP1066 administered at 30 and 90 minutes after CCI partially inhibited STAT3 phosphorylation in injured hippocampus 3, 6 and 24 hours after CCI.

d. Finally, we administered WP1066 75 mg/kg ip 30 and 90 minute post-CCI and harvested tissue at multiple timepoints after CCI. These studies demonstrated that WP1066 at this dose administered at 30 and 90 minutes after CCI more significantly inhibited STAT3 phosphorylation in injured hippocampus 6, 24 and 48 hours after CCI (see **Figure 15**).

Supporting Data: **Figures 13-15**

1c. Measure levels of mRNA for JaK/STAT, ICER and *Gabr* subunits in brain regions ipsilateral and contralateral to injury using qRT-PCR (30 mice [subset of mice as in Task1b]; months 12-15).

Status: Completed

Accomplishments: α1 expression is decreased in both CCI-S and CCI-S + WP injured mice 6 hours, 24 hours, and 48 hours after CCI.

Supporting Data: **Figure 16**

1d. Measure protein levels of JaK/STAT, ICER and *Gabr* subunits using western blotting in brain regions (30 mice [subset of mice as used in Task 1b]; months 15-18).

Status: Completed
Accomplishments:
1. Demonstrated that 30 and 90 minute post-treatment with WP1066 inhibits phosphorylation of STAT3 in injured hippocampus after both moderate and severe CCI at 6, 24 and 48 hours after CCI (**Figure 15**).
2. Demonstrated that 30 and 90 minute post-treatment with WP1066 rescues changes in alpha1 GABAAR subunits in injured hippocampus after severe CCI at 24 hours, 48 hours, 72 hours and 1 and 16 weeks after CCI. We also demonstrated that there are differential decreases in GABAAR alpha 1 subunit between the CCI-M and CCI-S injured mice at 24, 48 and hours and 1 and 16 weeks after injury (**Figure 17**).
3. $GABA_A$ receptor subunit $\gamma 2$ is decreased while δ, $\beta 3$ and $\beta 3$ are unchanged 1 and 16 weeks post CCI-S and administration of WP1066 does not affect these $GABA_AR$ subunit levels (**Figure 18**).

Supporting Data: **Figures 15, 17, 18**

1e. Assess protein levels and regional/cellular expression of JaK/STAT, ICER and *Gabr* subunits using fluorescent immunohistochemistry (30 mice [subset of mice used in Task 1b]; months 18-21).
Status: Not completed
Accomplishments: Not completed
Supporting Data: None

1f. Assess cell injury and neurogenesis using Fluoro-Jade, TUNEL, caspase and nestin staining; (30 mice [same mice used in Task 1e] months 18-21).
Status: Not completed
Accomplishments: Not completed
Supporting Data: None

1g. Assess glial proliferation using GFAP and ALDH1L1 staining (30 mice [same mice used in Task 1e]; months 18-21).
Status: Not completed
Accomplishments: Not completed
Supporting Data: None

1h. Implant and video-EEG monitor a subset of mice from 6-10 weeks post injury (80 mice - assuming 30% loss due to death with surgery or loss of implanted recording electrodes; months 16-20)
Status: Completed
Accomplishments:
1. Established collaborative relationship with University of Colorado Small Animal Neurophysiology core facility, an electrically clean facility on campus for long-term rodent video-EEG monitoring
2. Completed training of study personnel in EEG electrode implantation
3. Optimized EEG electrode implantation protocol for CCI animals. Because the skull and the underlying brain is disrupted during CCI, these animals require a specialized implantation protocol to minimize the impact of the skull and brain disruption on the quality of the collected signal (see **Figure 19**).
4. Interictal spiking density is unchanged between CCI-S, CCI-M and sham injured mice 11 weeks post injury (**Figure 20**).
5. Quantitation of interictal spiking density showed no differences between sham injured controls, CCI-S injured mice and CCI-S+WP injured mice. Quantitation of interictal spiking showed a significant increase in spiking in CCI-S+WP epileptic mice compared to CCI-S+WP mice that did not develop epilepsy (**Figure 21**).

6. No statistically significant differences in PTE development were found between any of the groups. The only group that had epileptic animals was the CCI-S injury group (**Table 2, Figure 22**).

Supporting Data: **Figures 19-22, Table 2**

1i. Assess mossy fiber sprouting, cell loss and glial proliferation 10 weeks post injury using Timm and Nissl staining (20 mice [subset of mice used in Task 1h]; months 20-21).
Status: Not completed
Accomplishments: Not completed
Supporting Data: None

1j. Assess pSTAT3, ICER and *Gabra1* protein levels 16 weeks post injury (20 mice [subset of mice used in Task 1h]; months 20-21).
Status: Completed
Accomplishments: See **Aim1** above.
Supporting Data: See **Figure 3.**

1k. Assess ICER and *Gabra1* mRNA levels 10 weeks post injury (20 mice [subset of mice used in Task 1h]; months 20-21).
Status: Completed
Accomplishments: See Aim 1 above.
Supporting Data: **Figure 2**

Task 2: Determine whether inhibition of STAT3 phosphorylation after CCI using shRNAs inhibits PTE development (Timeframe months 21-29)

Status: Not initiated.
Accomplishments: None
Supporting Data: None

The overall goal of the shRNA studies in Task 2 is to examine the effects of modulation of JaK/STAT pathway activation on epileptogenesis after experimental TBI using a second independent method. However, extensive background work we performed in the first year of the funding period in preparation for the planned studies showed that the vector we proposed to use for shRNA introduction (lentivirus) is selective for neural progenitor and glial cells only, making the lentiviral delivery of shRNAs using currently available vextors, less useful for our planned epileptogenesis studies that are focused on changes in neuronal excitability. These findings suggested that the shRNA studies, as planned, were not feasible for the proposed studies and would not add significantly to the body of findings generated during this project. Instead, our lab executed additional experiments with a more severe injury to take the place of the above task that are also designed to vary the severity of the experimental TBI and assess for alterations in JaK/STAT activation.

Task 3: Determine whether inhibition of STAT3 phosphorylation with WP1066 in animals with PTE reduces seizure frequency and/or inhibits PTE progression (Timeframe months 30-36)

3a. Induce TBI using the CCI model in adult CD-1 mice (50 mice- assuming 25-30% loss due to death or suboptimal injury; months 30-31)
Status: Completed. See Aim 1-1a above.

3b. Implant with subdural electrodes and video-EEG monitor mice from 6-8 weeks post injury as baseline (40 mice [assuming 30% loss due to death with surgery or loss of implanted recording electrodes]; months 32-34)
Status: Completed.
Accomplishments:
Supporting Data: Figure 19, 22.

3c. Administer WP1066 50-100 mg/kg or vehicle daily for 2 weeks and continue video- EEG monitoring (30 mice; months 32-35)

Status: Not completed
Accomplishments: Not completed
Supporting Data: None

There was no clear effect on seizure outcome from acute administration of WP1066. The fact that the effect of WP1066 delivered acutely is known to last through the changes seen in STAT3 phosphorylation after injury is suggestive of an ultimate lack of effect even when WP1066 is delivered chronically.

3d. Sacrifice animals and assess protein and mRNA levels for Gabr subunits and perform histological assessment of cell counts and mossy fiber sprouting (30 mice [same mice used in 3c]; months 34-36)
Status: Completed
Accomplishments: Receptor protein levels assessed at the chronic time point. See Aim1 above.
Supporting Data: See Figure 2 and 3.

An additional aspect of any intervention to prevent the development of epilepsy after TBI is to ensure that the intervention itself does not hamper recovery from the injury. Our lab has used two behavioral tests-
an accelerating rotorod test and a novel object recognition test- to assess motor and memory function pre-injury and at 3 days and 7 days post-injury in injured animals and plan to compare these results to those from WP1066-treated animals.
Accomplishments:
1. Injured animals reliably display injury-related declines in function on both tests, with the more severely injured animals performing worse than the moderately injured animals.
2. Memory performance is not significantly changed while vestibular motor performance is partially rescued with administration of WP1066.

Supporting Data: See **Figures 23 and 24.**

KEY RESEARCH ACCOMPLISHMENTS:

- Refined precise injury parameters for CCI in mice that yield consistent and reliable outcome measures. All tasks required the CCI model to be established. Determined that 1 mm injury was necessary and sufficient to induce cellular and behavioral changes associated with epileptogenesis.
- Determined that JaK2 and pSTAT3 are significantly increased in injured mouse hippocampus after CCI of both moderate and severe severity. In addition, pSTAT3 levels are significantly greater in the more severely injured animals suggesting that the JaK/STAT pathway may be differentially activated based on injury severity, and may potentially contribute to the differential sensitivity to post-traumatic epilepsy seen in humans with varying severities of TBI.
- Demonstrated that GABAAR subunit protein level α1 is significantly decreased at 12, 14 and 16 weeks after severe CCI. Also, the GABAAR subunits α2, α5, β2, and β3 protein levels are not significantly changed in injured hippocampus 12, 14, 16 weeks after severe CCI.
- Demonstrated that treatment with WP1066 75 mg/kg ip at 30 and 90 minute post-CCI inhibits phosphorylation of STAT3 in injured hippocampus 3, 6 and 24 hours after CCI. This establishes feasibility of using WP1066 to examine the effects of inhibition of pSTAT3 on epilepsy development after CCI in mouse.

- Demonstrated that 30 and 90 minute post-treatment with WP1066 rescues changes in alpha1 GABAAR subunits in injured hippocampus after severe CCI at 24 hours, 48 hours, 72 hours and 12-16 weeks after CCI. We also demonstrated that there are differential decreases in GABAAR alpha 1 subunit between the CCI-M and CCI-S injured mice at 24 hours and 12, 14, and 16 weeks after injury.
- Demonstrated that, in mouse, mossy fiber sprouting in the inner molecular layer is regionally and locally enhanced after CCI in a semi-quantitatively measurable manner. JAK/STAT3 inhibition did not affect mossy fiber sprouting 6-10 weeks post-injury.
- Selective hilar GABA interneuron loss was documented near the injury site after CCI-injury. JAK/STAT3 inhibition did not alter the cell loss.
- Total tonic GABA currents were not significantly altered in granule cells of the dentate gyrus ipsilateral to the injury 6-10 weeks after CCI in mice. However, THIP-induced tonic currents were reduced ipsilateral to the injury by 6-10 weeks after CCI-injury. Inhibition of STAT3 with WP1066 at the time of CCI-injury did not reinstate the THIP-activated tonic current ipsilateral to the injury.
- Zolpidem induced a significant increase in IPSC frequency in neurons from all groups at both the 1-6 week and 10 week time points. Inhibition of STAT3 with WP1066 at the time of CCI-injury did not alter the effect of zolpidem on IPSCs. The effect was likely due to network interactions among GABAergic inhibitory neurons.
- Zolpidem induced a significant increase in tonic current in neurons from CCI-injured mice compared to controls at both the 1-6 week and 10 week time points.
- Inhibition of JAK/STAT3 with WP1066 at the time of CCI-injury abrogated the effect of zolpidem on tonic currents.
- Demonstrated that JAK/STAT inhibition post-injury did not reduce development of post-traumatic epilepsy or memory impairment.
- Demonstrated that JAK/STAT inhibition post-injury improved motor recovery as assessed by RotaRod testing.

REPORTABLE OUTCOMES: manuscripts, abstracts, presentations

1. Hunt, R.F., Haselhorst, L.A., Schoch, K.M., Bach, E.C., Rios-Pilier, J., Scheff, S.W., Saatman, K.E., and Smith, B.N. (2012) Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains. *Epilepsy Res.* 99:167-170.
2. Butler CR, Boychuk, JA, Raible, DJ, Frey, L, Brooks-Kayal, AR, and Smith BN (2012) JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice. *Soc. Neurosci. Abs.*, 38:247.03
3. Boychuk JA, Butler, CR, Raible, DJ, Frey, L, Brooks-Kayal, AR, and Smith BN (2012) Focal traumatic brain damage results in localized GABA neuron loss and JAK/STAT activation early following injury. *Epilepsy Curr*. 13: 53:2.007.
4. D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. Rocky Mountain Regional Neuroscience Group Annual Meeting, UC AMC. 2013
5. D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. CTSA national pre-doctoral meeting. 2013
6. D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Poster Presentation. Department of Neurology Research Retreat. 2013

7. D Raible, L Frey, J Boychuk, C Butler, H Grabenstatter Y Cruz Del Angel, S Russek, B Smith and A Brooks-Kayal. JaK/STAT inhibition to prevent posttraumatic epilepsy. Oral Presentation. Rocky Mountain Regional Neuroscience Group Annual Meeting. 2013
8. Butler, CB, Boychuk, JA, Frey, L, Brooks-Kayal, AR, and Smith, BN. Inhibitory signaling to dentate granule cells following traumatic brain injury. *Soc. Neurosci. Abs.*, 39: 536.08; 2013.
9. Raible DJ, Boychuk J, Butler C, Cruz Del Angel Y, Russek SJ, Smith BN, Frey L, Brooks-Kayal AR. JaK/STAT Inhibition to Prevent Post-Traumatic Epileptogenesis. American Epilepsy Society Annual Meeting, Abstract and Poster presentation, Washington DC, 2013.
10. Hall, E.D., Smith, B.N., Brooks-Kayal, A., and Soltesz, I. (2014) When ski helmets aren't enough: emerging therapies for TBI and post-traumatic epilepsy. *Winter Conf. Brain Res.*
11. Raible DJ, Frey LC, Cruz Del Angel Y, Carlsen J, Hund D, Smith BN, Brooks-Kayal AR. $GABA_A$ Receptor Regulation after differing severities of CCI and modulation of JAK/STAT pathway activation with WP1066. In Preparation.

CONCLUSIONS

The CCI model was refined in mice at both University of Colorado and University of Kentucky. Essential baseline control data was obtained to ensure that all aspects of CCI-injury and of WP1066 treatment were feasible and repeatable between laboratories. Notably, 1 mm injury depth resulted in STAT phosphorylation, inhibitory cell loss, mossy fiber sprouting, and reduction in THIP-activated tonic GABA currents. A significant percentage of these animals were confirmed to express behavioral seizures at University of Kentucky. At the University of Colorado, we had success with a 2 mm injury, so effects of this more severe injury on the parameters were studied to determine if an injury severity-response relationship exists.

We have demonstrated that JaK2 and pSTAT3 are significantly increased in injured mouse hippocampus after CCI of both moderate and severe severity, and that pSTAT3 levels are significantly greater in the more severely injured animals suggesting that the JaK/STAT pathway may be differentially activated based on injury severity, and may potentially contribute to the differential sensitivity to post-traumatic epilepsy seen in humans with varying severities of TBI. We have further shown that protein levels of GABAAR α1 subunit, but not other GABAAR subunits, are significantly decreased at multiple chronic timepoints after severe CCI. We have also demonstrated that treatment with WP1066 75 mg/kg ip at 30 and 90 minute post-CCI inhibits phosphorylation of STAT3 in injured hippocampus 3- 24 hours after CCI, and that this treatment prevents CCI-induced reductions in alpha1 GABAAR subunits in injured hippocampus after severe CCI at all acute and chronic timepoints. Select GABA neuron loss is seen shortly after injury, and mossy fiber sprouting develops after several weeks post-injury, neither of which outcome was altered in WP1066-treated mice. We further did not find evidence that JAK/STAT inhibition with WP1066 reduced the frequency of psot-traumatic epilepsy development, nor did it signficantly improve psot-injury memory function on Novel Object Recognition testing (although there was a trend in this direction). We did see improved motor function in CCI animals treated with WP1066 compared to vehicle treated injured animals, suggesting that JAK./STAT inhibition may improve motor recovery. A manuscript reporting these findings is in final preparation and will be submitted in the next few weeks.

Total tonic GABA currents in granule cells are unaffected by the injury. However, contrary to some other models, THIP-activated tonic GABA currents are reduced in granule cells ipsilateral to the injury, suggesting a reduction in δ GABA receptor subunits (possibly α4/δ-containing), as hypothesized. Treatment with WP1066 did not reinstate the reduction in THIP-activated current, suggesting that the decrease in δ-subunit containing GABA receptors after injury is not affected by JAK/STAT3 inhibition.

Previously, Raible et al (2012) showed that α4-subunits are decreased one week after fluid percussion injury. Often, α4-subunits pair with δ-subunits. The reduction in THIP-activated current is consistent with this finding. It is also consistent with a lack of influence of JAK/STAT3 phosphorylation on δ-subunit expression. Specific α4-subunit associated effects were the target of furosemide experiments. However, since furosemide had only minor effects on tonic currents, and the drug was determined to have numerous effects not related to α4-subunit modulation, results of experiments using furosemide were not conclusive. Alterations in α1 subunit function were targeted using zolpidem, a relatively selective benzodiazepine-like agonist. Zolpidem application resulted in a network effect on GABAergic synaptic connectivity, which was statistically enhanced in CCI-injured mice, but was not affected by treatment with JAK/STAT3 blocker. Notably, zolpidem also induced a significantly larger GABAergic tonic current in dentate granule cells from CCI-injured mice, and this effect was eliminated in neurons from mice that were treated with the JAK/STAT3 inhibitor. It is possible that CCI-injury results in increased α1 subunit expression selectively in granule cells, and this expression is modulated by JAK/STAT3. It is also possible that the injury induces de-novo expression of α1,2,3 or 5 subunits in granule cells. Additional experiments are ongoing to resolve this issue. A manuscript will be submitted for publication later this year, which describes GABA current changes and effects of WP1066 after CCI injury.

REFERENCES

Butler CR, Boychuk, JA, Raible, DJ, Frey, L, Brooks-Kayal, AR, and Smith BN (2012) JAK/STAT Activation and GABA Neuron Loss After Focal Traumatic Brain Injury in Mice. *Soc. Neurosci. Abs.*, 38:247.03

Boychuk, J.A., Butler, C.R., Raible, D., Frey, L., Brooks-Kayal, A., and Smith, B.N (2012) JAK/STAT Activation and GABA neuron loss after focal traumatic brain Injury in mice. *Epilepsia*. 53:2.007.

Hunt, R.F., Scheff, S.W., and Smith, B.N. (2009) Posttraumatic epilepsy after controlled cortical impact injury in mice. *Exp. Neurol.* 215(2):243-52.

Hunt, R.F., Haselhorst, L.A., Schoch, K.M., Bach, E.C., Rios-Pilier, J., Scheff, S.W., Saatman, K.E., and Smith, B.N. (2012) Posttraumatic mossy fiber sprouting is related to the degree of cortical damage in three mouse strains. *Epilepsy Res.* 99:167-170.

Raible DJ, Frey LC, Cruz Del Angel Y, Russek SJ, Brooks-Kayal AR. (2012) GABA(A) receptor regulation after experimental traumatic brain injury. J Neurotrauma. 2012 Nov 1;29(16):2548-54.

APPENDIX

Item #1- Hall, E.D., Smith, B.N., Brooks-Kayal, A., and Soltesz, I. (2014) When ski helmets aren't enough: emerging therapies for TBI and post-traumatic epilepsy. *Winter Conf. Brain Res.*

SUPPORTING DATA:

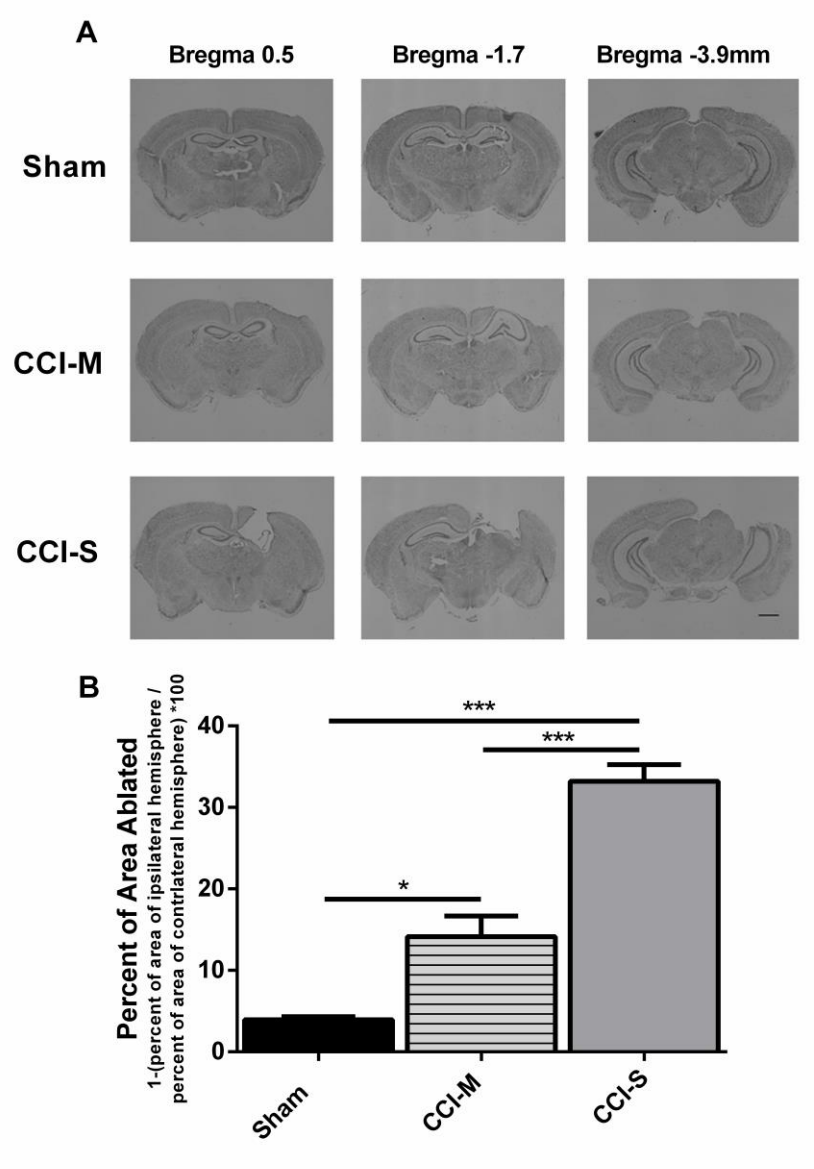

Figure 1. Progressive tissue loss following CCI-S and CCI-M injuries. (A) A series of representative images of CV stained coronal sections from sham, CCI-S and CCI-M injured mice. Sections show cerebral damage at three different levels posterior from bregma. Calibration bar, 5 mm (B) Quantitation of lesion extent by measuring the area of the ipsilateral hemisphere and normalizing it to the area of the contralateral hemisphere for sham, CCI-S and CCI-M injured mice. Each group is statistically different from each other group with CCI-S having the largest lesion. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ ($n = 4$ for sham, $n = 7$ for CCI-M and $n = 8$ for CCI-S)

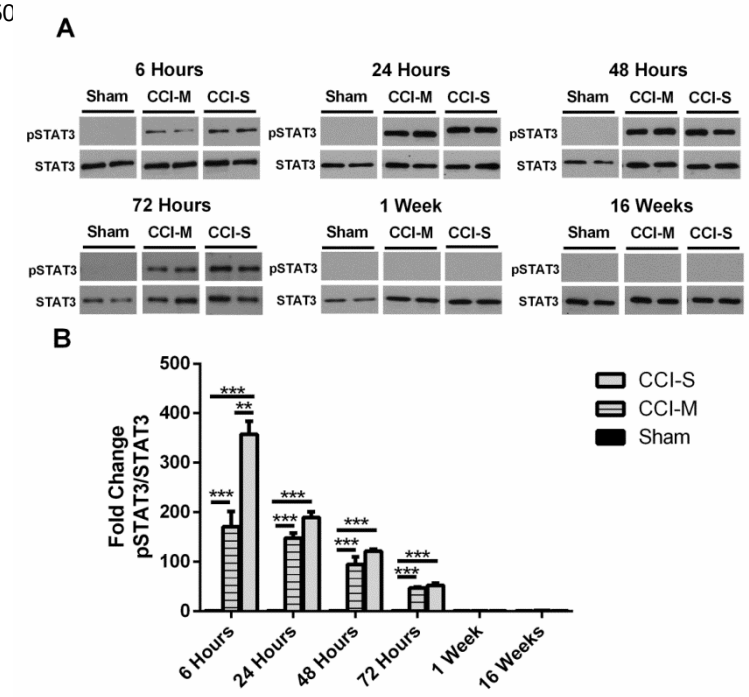

Figure 2. Phosphorylated STAT3 levels in injured hippocampus after CCI. (A) Representative western blots of protein homogenates from whole ipsilateral hippocampus (relative to CCI) of mice 6 hours, 24 hours, 48 hours, 1 week and 16 weeks after CCI probed with pSTAT3 and STAT3 antibodies. (B) Quantification of pSTAT3 levels from CCI-S, CCI-M and sham injured controls showed that from 6 hours to 72 hours post injury that pSTAT3 levels were higher in both the CCI-S and CCI-M injured group when compared to sham injured controls. CCI-S pSTAT3 levels were statistically higher than CCI-M levels at 6 hours post injury but, at all other time points there was no statistical difference between CCI-S and CCI-M pSTAT3 protein levels. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ ($n = 9$ for sham, $n = 9$ for CCI-S and $n = 9$ for CCI-M at 6 hours, $n = 10$ for sham, $n = 9$ for CCI-S and $n = 10$ for CCI-M at 24 hours and $n = 7$ for sham, $n = 7$ for CCI-S and $n = 7$ for CCI-M at 48 hours and $n = 6$ for sham, $n = 8$ for CCI-S and $n = 6$ for CCI-M at 72 hours and $n = 6$ for sham, $n = 7$ for CCI-S and $n = 7$ for CCI-M at 1 week and $n = 6$ for sham, $n = 7$ for CCI-S and $n = 6$ for CCI-M at 16 weeks).

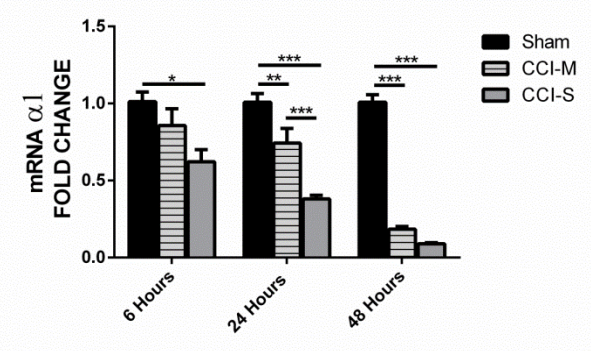

Figure 3. α1 expression is decreased in both CCI-S and CCI-M injured mice 6 hours, 24 hours, and 48 hours after CCI. mRNA levels of α1 were quantified using RT-PCR analysis and represented as histograms showing the fold change of α1 6 hours, 24 hours and 48 hours after CCI in CCI-S, CCI-M and sham injured controls. α1 mRNA levels were normalized to B2M mRNA levels in the same samples and expressed as a fold change compared to sham injured controls (defined as 1). * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ (n = 7 for sham, n = 7 for CCI-S and n = 6 for CCI-M at 6 hours and n = 7 for sham, n = 7 for CCI-S and n = 7 for CCI-M at 24 hours and n = 7 for sham, n = 7 for CCI-S and n = 7 for CCI-M at 48 hours).

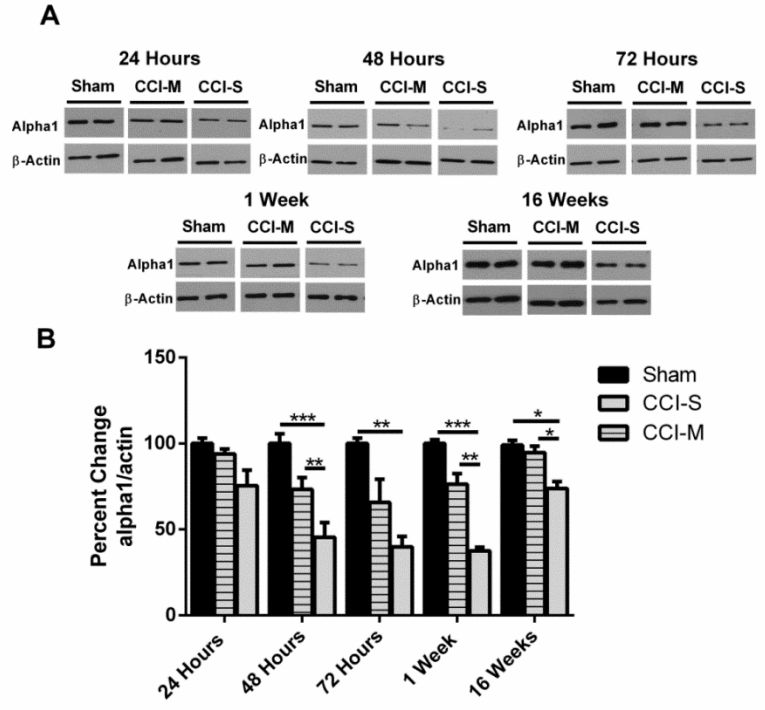

Figure 4. Temporal profile of $GABA_A$ α1 receptor subunit after CCI.
(A) Representative western blots form whole hippocampus of mice 24 hours, 48 hours, 72 hours, 1 week and 16 weeks after CCI probed with anti-$GABA_AR$ α1 and β-actin antibodies. (B) Quantification of α1 blots showed a significant decrease at 48 hours through 16 weeks after CCI-S injury relative to CCI-M and sham injured controls. α1 levels were normalized to β-actin levels and expressed as a percent change compared to sham injured controls. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ (n = 7 for sham, n = 7 for CCI-S and n = 6 for CCI-M at 24 hours and n = 7 for sham, n = 7 for CCI-S and n = 7 for CCI-M at 48 hours and n = 6 for sham, n = 6 for CCI-S and n = 6 for CCI-M at 72 hours and n = 6 for sham, n = 7 for CCI-S and n = 7 for CCI-M at 1 week and n = 7 for sham, n = 10 for CCI-S and n = 9 for CCI-M at 16 weeks).

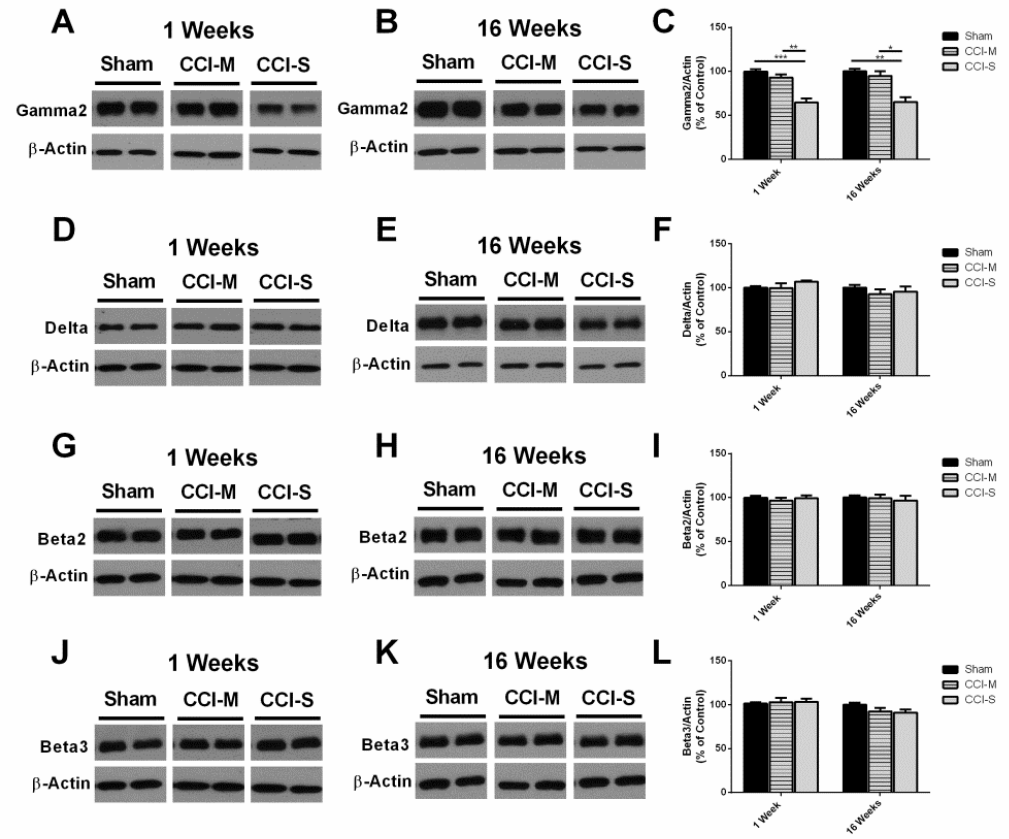

Figure 5. $GABA_A$ receptor subunit γ2 is decreased while δ, β3 and β3 are unchanged 1 and 16 weeks post CCI.

(A) Representative western blots from whole hippocampus of mice 1 week after CCI probed with anti-$GABA_AR$ γ2 and β-actin antibodies. (B) Representative western blots from whole hippocampus of mice 16 weeks after CCI probed with anti-$GABA_AR$ γ2 and β-actin antibodies. (C) Quantification of γ2 blots showed that the γ2 subunit is significantly decreased 1 and 16 weeks after CCI-S relative to CCI-M and sham injured controls. (D) Representative western blots from whole hippocampus of mice 1 week after CCI probed with anti-$GABA_AR$ δ and β-actin antibodies. (E) Representative western blots from whole hippocampus of mice 16 weeks after CCI probed with anti-$GABA_AR$ δ and β-actin antibodies. (F) Quantification of δ blots showed that the δ subunit was unchanged 1 and 16 weeks after CCI-S relative to CCI-M and sham injured controls. (G) Representative western blots from whole hippocampus of mice 1 week after CCI probed with anti-$GABA_AR$ β2 and β-actin antibodies. (H) Representative western blots from whole hippocampus of mice 16 weeks after CCI probed with anti-$GABA_AR$ β2 and β-actin antibodies. (I) Quantification of β2 blots shows that the β2 subunit was unchanged 1 and 16 weeks after CCI-S relative to CCI-M and sham injured controls. (J) Representative western blots from whole hippocampus of mice 1 week after CCI probed with anti-$GABA_AR$ β3 and β-actin antibodies. (K) Representative western blots from whole hippocampus of mice 16 weeks after CCI probed with anti-$GABA_AR$ β3 and β-actin antibodies. (L) Quantification of β3 blots showed that the β3 subunit was unchanged 1 and 16 weeks after CCI-S relative to CCI-M and sham injured controls. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ (n = 6 for sham, n = 7 for CCI-S and n = 7 for CCI-M at 1 week and n = 10 for sham, n = 10 for CCI-S and n = 9 for CCI-M at 16 weeks).

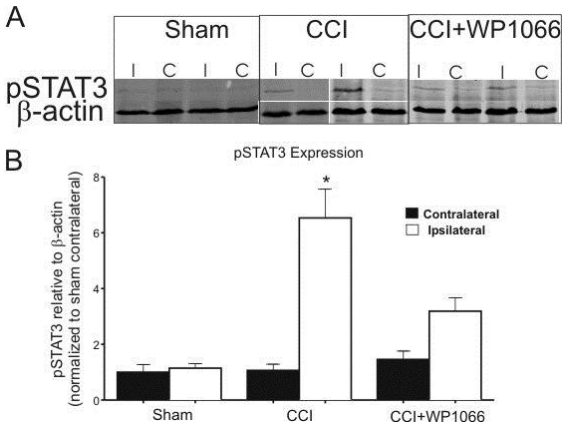

Figure 6. **Western blot and representative bar graph of pSTAT3 expression relative to β-actin content in CCI-injured, sham- injured, and CCI-injured + WP1066 treatment.** Expression of pSTAT3 is significantly increased in the ipsilateral hemisphere of CCI hippocampus compared to Sham 24 hr after injury. Expression of pSTAT3 in mice injected with the STAT3 inhibitor WP1066 is significantly reduced relative to CCI ($P<0.05$) and is similar to sham levels ($P>0.05$; Tukey's ANOVA post hoc).

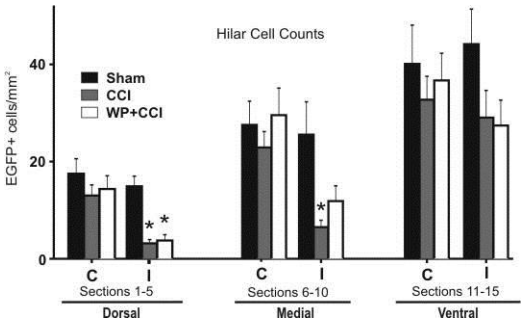

Figure 7. Hilar interneuron counts in Sham, CCI, and CCI with WP1066 treatment. Sections were taken in a 1 in 5 series at 30 µm. Sections were divided into dorsa,l medial, and ventral portions (5 section per group). Cell density in dorsal hippocampus after CCI and CCI+WP1066 treatment were significantly decreased compared to Sham in the ipsilateral hemisphere (Tukey's ANOVA post hoc; $p<0.05$). In medial hippocampus, only CCI was significantly decreased compared to Sham ipsilateral hemisphere There were no differences in ventral hippocampus. There were no significant differences between groups in contralateral hemisphere or between CCI and CCI with WP1066 treatment in the ipsilateral hemisphere.

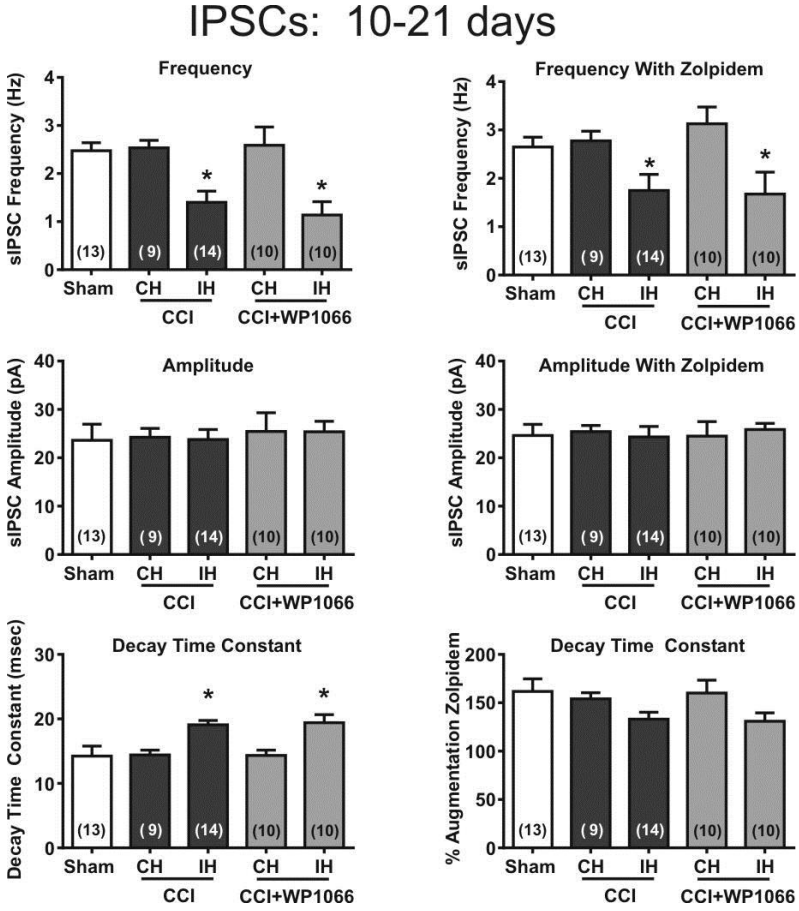

Figure 8. Graphical illustration of zolpidem effects on IPSCs 10-21 days post-injury. Frequency - Prior to zolpidem application, experimental groups exhibited a significant main effect of sIPSC frequency [$F(4,51) = 7.65$, $p < 0.0001$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI or CCI+WP1066 animals had significantly lower sIPSC frequencies compared to Sham controls (Tukey's, $p= 0.013$ and $p= 0.0034$, respectively) or their contralateral cell counterparts controls (Tukey's, $p= 0.021$ and $p= 0.0027$, respectively). Ipsilateral cells from CCI versus CCI+WP1066 animals did not significantly differ in sIPSC frequency under baseline conditions (Tukey's, $p= 0.94$). A significant main effect of sIPSC frequency was also detected in the presence of zolpidem [$F(4,51) = 4.023$, $p= 0.0065$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI or CCI+WP1066 animals had significantly lower sIPSC frequencies compared to contralateral cells from the WP1066 group (Tukey's, $p= 0.024$ and $p= 0.030$, respectively) but not from Sham control (Tukey's, $p= 0.20$ and $p =0.21$) or the contralalteral CCI group (Tukey's, $p= 0.18$ and $p= 0.19$). **Amplitude** - No significant main effect of condition was detected between groups in the amplitude of sIPSC under baseline conditions [$F(4,51) = 0.095$, $p= 0.98$] or in the presence of zolpidem [$F(4,51) = 0.088$, $p= 0.99$]. **Decay Time Constant** - A significant main effect of condition was detected between for sIPSC decay time constant in the absence of zolpidem [$F(4,51) = 6.03$, $p= 0.0005$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI animals possessed a significantly greater sIPSC decay time constant compared to Sham controls (Tukey's, $p= 0.011$) or their contralateral cell counterpart controls (Tukey's, $p= 0.037$). Similarly, DGCs ipsilateral to injury from CCI+WP1066 animals possessed a significantly greater sIPSC decay time constant compared to Sham controls (Tukey's, $p= 0.014$) or their contralateral cell counterpart controls (Tukey's, $p= 0.028$). A post hoc comparison of ipsilateral DGCs from CCI versus CCI+WP1066 animals detected no difference in sIPSC decay time constant (without zolpidem) for these groups (Tukey's, $p > 0.99$). In contrast, a comparison of zolpidem's percent augmentation of decay time constant revealed no significant main effects although a statistical trend was noted [$F(4,51) = 2.16$, $p= 0.087$]. A post hoc comparison of ipsilateral DGCs from CCI versus CCI+WP1066 animals detected no difference in zolpidem augmentation of sIPSC decay time constant for these groups (Tukey's, $p > 0.99$).

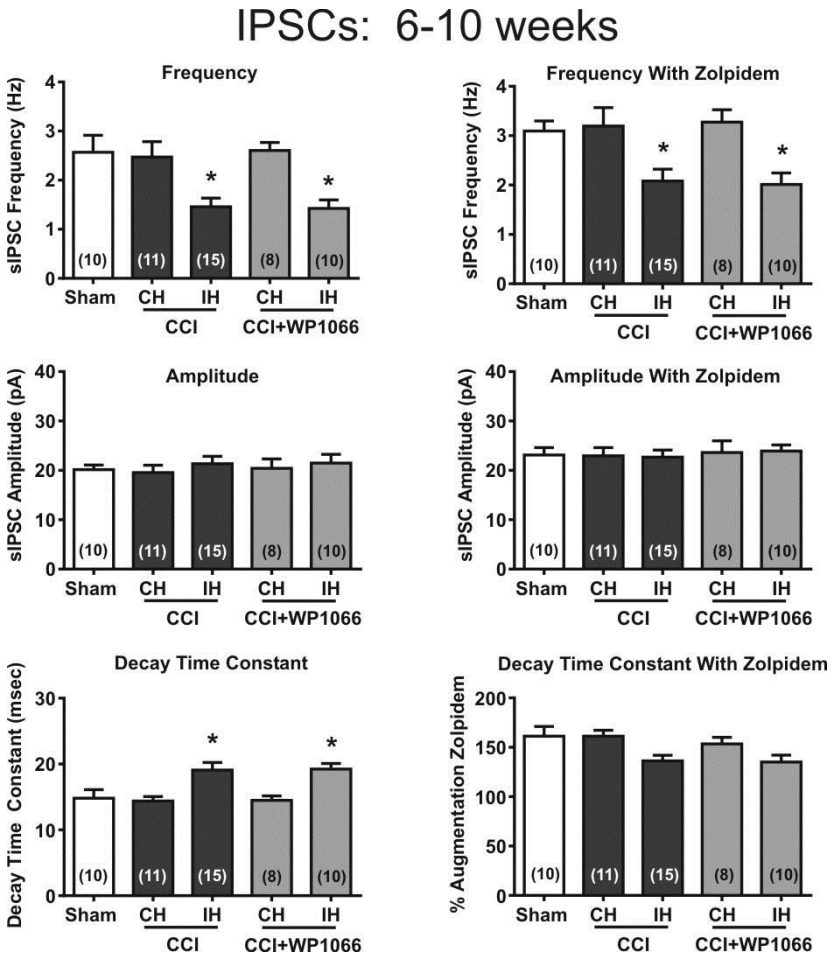

Figure 9. Graphical illustration of zolpidem effects on IPSCs 6-8 weeks post-injury. Frequency - Experimental groups exhibited a significant main effect of sIPSC frequency under pre-zolpidem conditions [$F(4,49) = 6.17$, $p= 0.0004$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI and CCI+WP1066 animals had significantly lower sIPSC frequencies compared to Sham controls (Tukey's, $p= 0.013$ and $p= 0.022$, respectively) or their contralateral cell counterparts controls (Tukey's, $p= 0.023$ and $p= 0.0028$, respectively). Ipsilateral cells from CCI versus CCI+WP1066 animals did not significantly differ in sIPSC frequency under these conditions (Tukey's, $p > 0.99$). A significant main effect of sIPSC frequency was also detected in the presence of zolpidem [$F(4,49) = 5.35$, $p= 0.0012$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI or CCI+WP1066 animals had significantly lower sIPSC frequencies compared to contralateral cells from their respective groups (CCI IH versus CCI CH, Tukey's, $p= 0.024$) and (CCI+WP1066 IH versus CCI+WP1066 CH, Tukey's, $p= 0.036$). A statistical trend for each injured group (ipsilateral CCI and ipsilateral CCI+WP1066) was detected relative to Sham controls (Tukey's, $p= 0.060$ and $p= 0.069$, respectively). DGCs ipsilateral to injury from CCI versus CCI+WP1066 animals exhibited sIPSC that did not significantly differ from each-other (Tukey's, $p > 0.99$). **Amplitude** - No significant main effect of condition was detected between groups in the amplitude of sIPSCs under zolpidem-free conditions [$F(4,49) = 0.29$, $p= 0.88$] or in the presence of zolpidem [$F(4,49) = 0.098$, $p= 0.98$]. **Decay Time Constant** - A significant main effect of condition was detected between groups for sIPSC decay time constant in the absence of zolpidem [$F(4,49) = 6.02$, $p= 0.005$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI animals possessed a significantly greater sIPSC decay time constants compared to Sham controls (Tukey's, $p= 0.029$) or their contralateral cell counterpart controls (Tukey's, $p= 0.010$). Similarly, DGCs ipsilateral to injury from CCI+WP1066 animals possessed significantly greater sIPSC decay time constants compared to Sham controls (Tukey's, $p= 0.044$) or their contralateral cell counterpart controls (Tukey's, $p= 0.039$). A post hoc comparison of ipsilateral DGCs from CCI versus CCI+WP1066 animals detected no difference in sIPSC decay time constant for these groups in the presence (Tukey's, $p > 0.99$) or absence of zolpidem (Tukey's, $p > 0.99$). A comparison of zolpidem's percent augmentation of decay time constant revealed a significant main effect [$F(4,49) = 3.59$, $p= 0.012$]. Based on multiple comparisons, neither ipsilateral DGCs from CCI animals nor ipsilateral DGCs from CCI+WP1066 animals presented significant differences in zolpidem augmentation of decay time constants relative to Sham or contralateral controls. There were however, statistical trends for reduced zolpidem augmentation of decay time constants for ipsilateral cells from CCI and CCI+WP1066 conditions relative to Sham controls (Tukey's, $p= 0.076$ and $p= 0.10$, respectively). A post hoc comparison of ipsilateral DGCs from CCI versus CCI+WP1066 animals detected no difference in zolpidem augmentation of sIPSC decay time constant for these groups (Tukey's, $p > 0.99$).

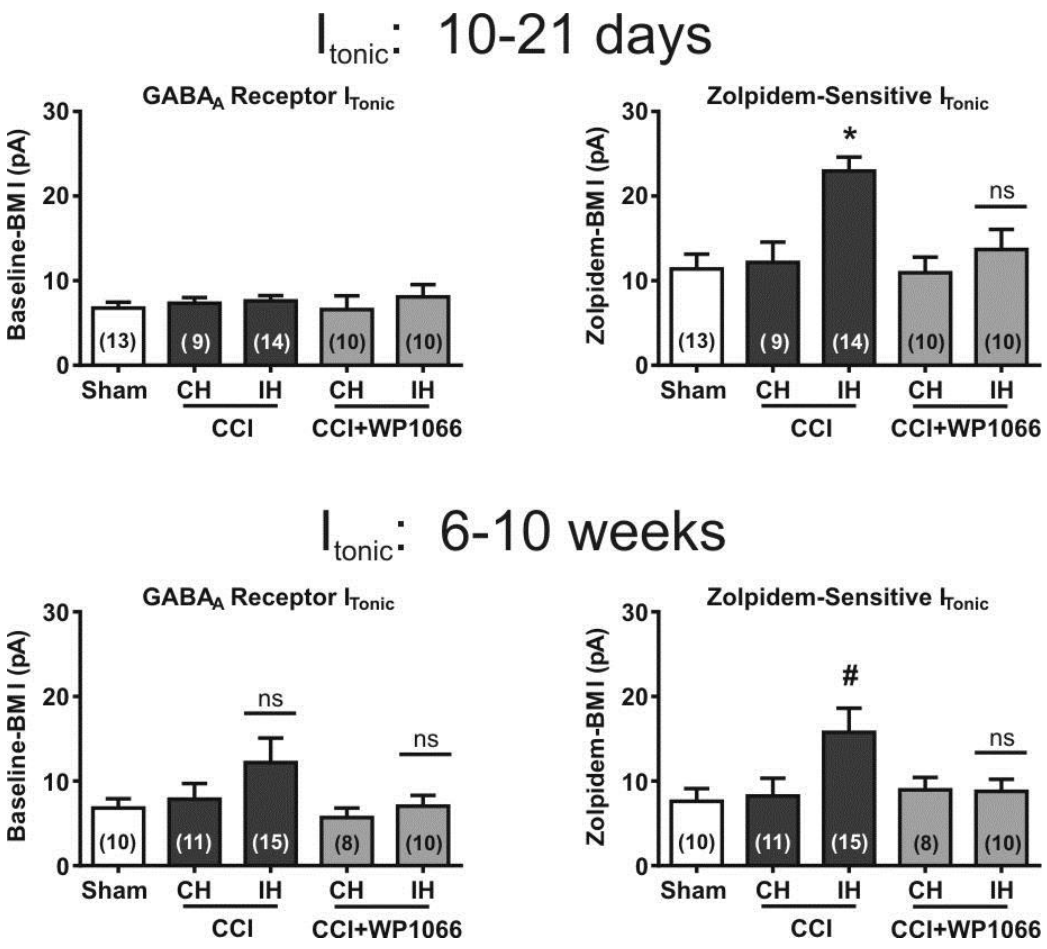

Figure 10. Graphical illustration of zolpidem on Itonic. Top, 10-21 days post-injury. Tonic GABA currents, in the absence of zolpidem, did not exhibit a significant main effect of condition between groups [$F(4,51) = 0.34$, $p= 0.85$]. In contrast, a significant overall effect on Tonic GABA currents in the presence of zolpidem was detected [$F(4,51) = 7.63$, $p < 0.0001$]. Multiple comparisons revealed that DGCs ipsilateral to injury from CCI animals possessed a significantly larger zolpidem-sensitive Tonic GABA current compared to Sham controls (Tukey's, $p= 0.0003$) or their contralateral cell counterpart controls (Tukey's, $p= 0.0028$). Interestingly, this zolpidem augmentation of the Tonic GABA current was absent in ipsilateral DGCs from CCI+WP1066 animals thereby indicating an effect of WP1066 (versus Sham controls, Tukey's, $p= 0.92$ and versus contralateral CCI+WP1066, Tukey's, $p= 0.88$). **Bottom,** 6-10 weeks post-injury. Tonic GABA currents, in the absence of zolpidem, did not exhibit a significant main effect of condition between groups [$F(4,49) = 1.60$, $p= 0.19$]. A significant overall effect on Tonic GABA currents in the presence of zolpidem was detected [$F(4,49) = 2.74$, $p=0.039$]. Subsequent multiple comparisons using Tukey's test identified no significant differences among individual conditions. However, statistical trends were observed for ipsilateral DGCs from CCI animals to possess larger zolpidem-sensitive Tonic GABA currents relative to Sham controls (Tukey's, $p= 0.069$) as well as their contralateral cell counterparts ($p= 0.095$). Ipsilateral cells from animals given CCI+WP1066 did not significantly differ from Sham controls (Tukey's, $p > 0.99$) or their contralateral cell counterparts ($p > 0.99$).

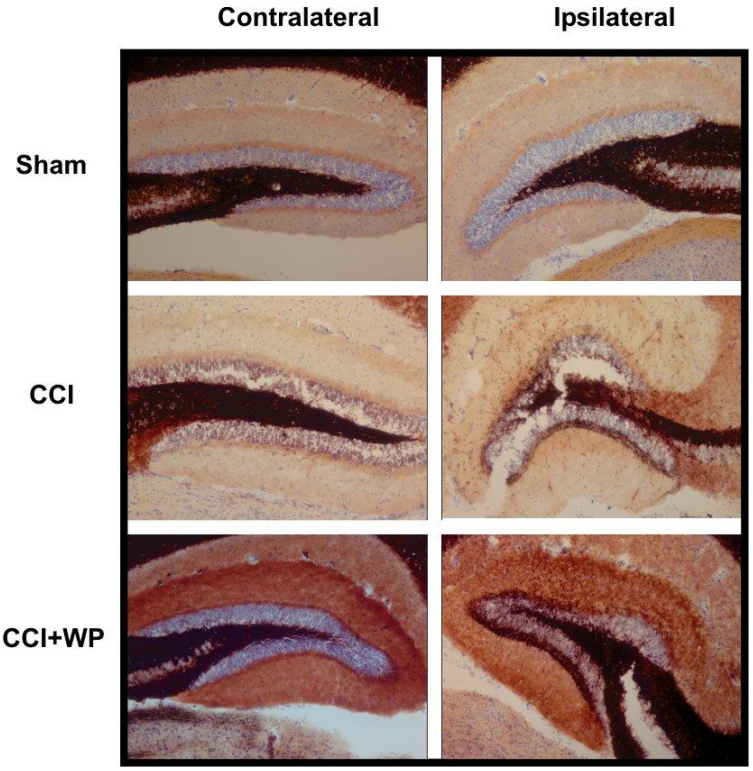

Figure 11. Timm staining in the dentate gyrus from sham control, CCI-injured, and CCI-injured+WP1066 treated mice. Images of the dentate gyrus contralateral (left images) and ipsilateral (right), directly under the 'epicenter' of the injury or skull opening in sham-operated controls, CCI-injured, or CCI-injured+WP1066-treated mice after ~8 weeks post-injury. Contralateral images are from approximately equivalent hippocampal levels contralateral to the skull opening. Mossy fiber sprouting into the inner molecular layer is not seen contralateral to the injury or skull opening. However, mossy fiber sprouting is evident ipsilateral to the injury in CCI-injured and CCI-injured+WP1066-treated mice. The degree of sprouting is similar for treated- and untreated CCI-injured mice and is similar to that seen previously to be associated with synaptic reorganization.

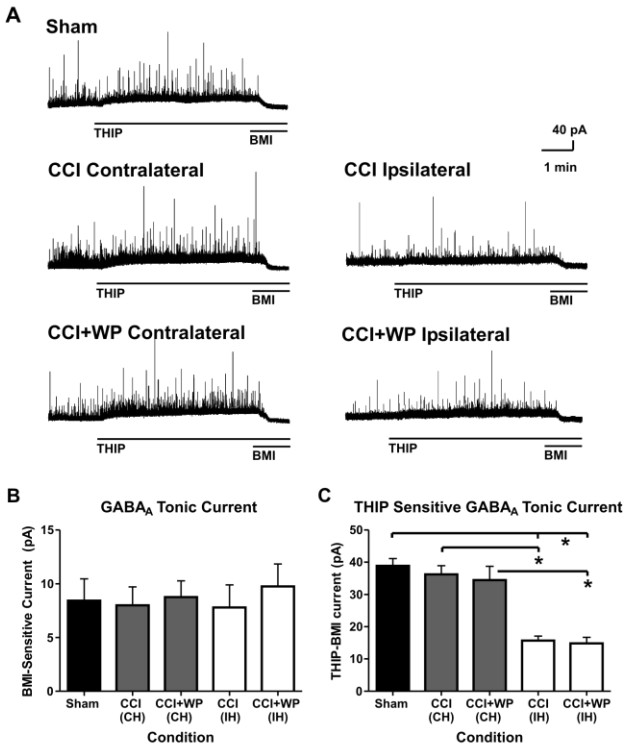

Figure 12. Reduced THIP-sensitive tonic $GABA_A$ currents in dentate granule cells (DGCs) located ipsilateral to controlled cortical impact (CCI) during 6-13 weeks post-injury. A. Representative traces of tonic $GABA_A$ currents in DGCs after sham injury (upper left; n=7), CCI injury (middle row; Contralateral: n=7, Ipsilateral: n=7) or CCI injury with acute WP1066 treatment (bottom row; Contralateral: n=5, Ipsilateral: n=7). DGCs were voltage clamped at 0 mV (near reversal potential of glutamatergic currents) and recorded in three phases: baseline, THIP (3 µM; Sigma, USA) and Bicuculline Methiodide (BMI; 30 µM; Tocris, USA). B. Group data of tonic $GABA_A$ currents measured as the change in steady-state holding current values of baseline to BMI application (Baseline I_{Hold} – BMI I_{Hold}). C. Group data of THIP-sensitive tonic $GABA_A$ currents measured as the change

in steady-state holding current values of THIP application to bicuculline application (THIP I_{Hold} – BMI I_{Hold}). Given the recoding parameters here, an increase in tonic GABA $_A$ receptor-mediated currents using THIP produced an outward shift whereas blockade of GABA $_A$ receptors with BMI produced an inward shift in the holding current. Data shown as mean ± SEM.

Table 1. IPSC frequency, amplitude and decay time constant in control, CCI-injured and CCI-injured+WP1066 treated mice; effects of THIP on IPSCs are also indicated.

sIPSCs	Sham	CCI				CCI+WP1066			
		Contralateral		Ipsilateral		Contralateral		Ipsilateral	
	Baseline	Baseline	THIP	Baseline	THIP	Baseline	THIP	Baseline	THIP
Frequency(Hz)	1.41±0.09	1.30±0.26	0.82±0.08	0.80±0.12*	0.68±0.08	1.33±0.34	0.74±0.35	0.72±0.09*	0.69±0.07
Peak Amplitude(pA)	20.36±1.92	20.89±1.88	22.11±3.05	22.85±3.02	20.93±2.24	21.72±2.58	23.34±3.36	18.71±1.57	21.22±1.78
10-90% Rise Time (ms)	2.23±0.10	2.29±0.18	2.24±0.44	2.47±0.11	2.53±0.18	2.30±0.13	2.33±0.09	2.45±0.18	2.42±0.12
Decay Time Constant (ms)	14.06±3.40	15.33±3.26	14.17±2.02	17.04±3.56	16.03±1.63	14.10±0.67	13.35±0.88	16.97±1.49	15.70±2.05

Synaptic responses were assessed at baseline and at steady-state during 4,5,6,7-Tetrahydroisoxazolo[5,4-c]pyridine-3-ol hydrochloride (THIP; 3 µM) application. Sample sizes are sham injury (n=7), CCI injury (Contralateral: n=7, Ipsilateral: n=7) or CCI injury with acute WP1066 treatment (Contralateral: n=5, Ipsilateral: n=7). Data shown as mean ± SEM. IPSC frequency was significantly reduced in CCI-injured mice relative to controls ($p<0.05$); WP1066 did not affect IPSC frequency.

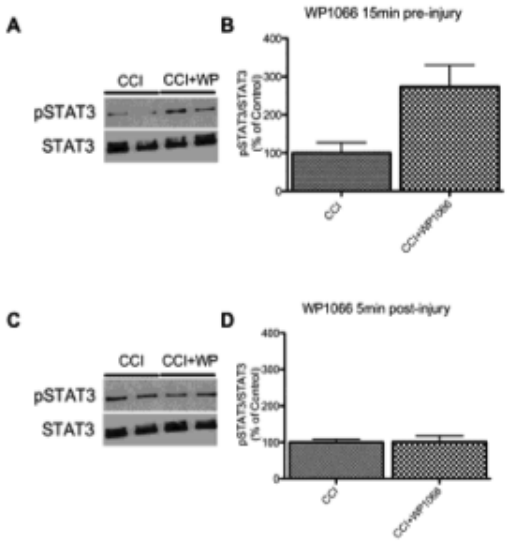

Figure 13: IP administration of WP1066 at 15 min before or 5 minutes after CCI did not inhibit phosphorylation of STAT3 in injured hippocampus 6 hours after CCI. (A,C) Representative western blots of protein homogenates from ipsilateral whole hippocampus (relative to CCI) of mice treated with WP1066 or DMSO (15 minutes prior to injury for A) or (5 minutes after injury for B) and sacrificed 6 hours after CCI probed with pSTAT3 and STAT3 antibodies. (B,D) Quantification of pSTAT3 levels from CCI + 50mg/Kg of WP1066 and CCI + DMSO mice. pSTAT3 levels were normalized to STAT3 levels and expressed as a percent change compared to sham controls. (15 minutes pretreatment n = 2 for CCI, 3 for CCI+wp1066; 5 minute post-treatment n = 2 for CCI, 3 for CCI+wp1066).

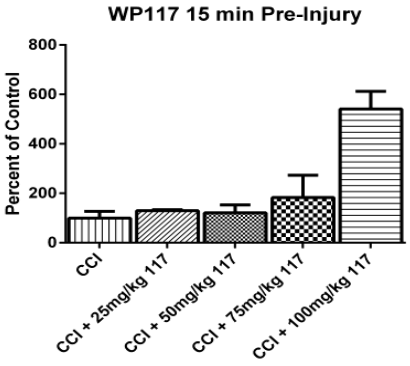

Figure 14: 15 minute pre-treatment IP administration of WP1066 analog (WP117) at varying does did not inhibit phosphorylation of STAT3 in injured hippocampus 1 hour after CCI. Quantification of pSTAT3 levels from CCI + 25mg/kg WP117, CCI + 50mg/kg WP117, CCI + 75mg/kg WP117, CCI + 100 mg/kg WP117 and CCI + DMSO mice. n=6 for CCI, n = 4 for CCI + 25mg/kg WP117; n = 7 for CCI +50mg/kg WP117; n = 2 for CCI + 75mg/kg WP117; n = 3 for CCI + 100mg/kg WP117.

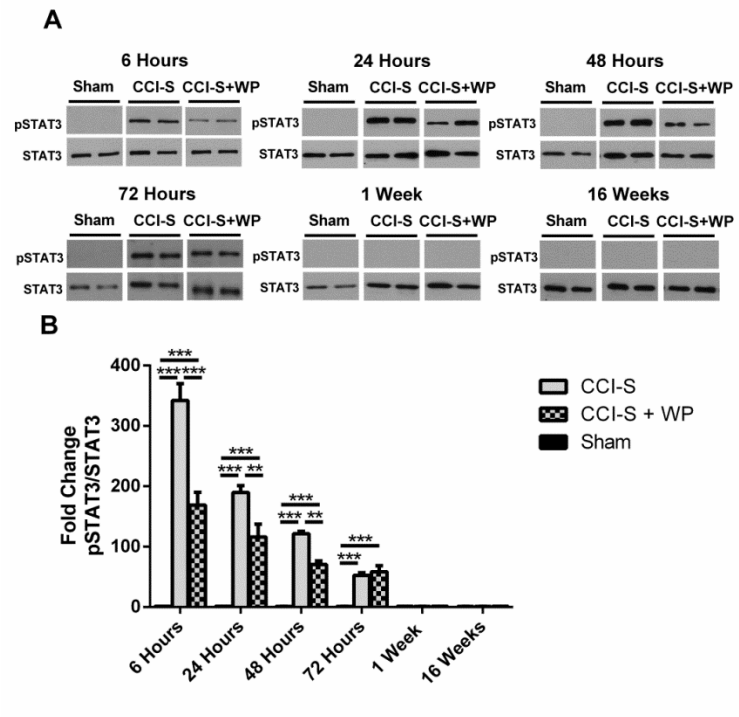

Figure 15. WP1066 administration after CCI-S reduces phosphorylated STAT3 levels in injured hippocampus.

(A) Representative western blots of protein homogenates from whole ipsilateral hippocampus (relative to CCI) of mice 6 hours, 24 hours, 48 hours, 1 week and 16 weeks after CCI probed with pSTAT3 and STAT3 antibodies. (B) Quantification of pSTAT3 levels from CCI-S, CCI-S + WP and sham injured controls showed that from 6 hours to 48 hours post injury that pSTAT3 levels were statistically lower than CCI-S injured mice. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ (n = 9 for sham, n = 9 for CCI-S and n = 6 for CCI-S + WP at 6 hours, n = 10 for sham, n = 9 for CCI-S and n = 7 for CCI-S + WP at 24 hours and n = 7 for sham, n = 7 for CCI-S and n = 7 for CCI-S + WP at 48 hours and n = 6 for sham, n = 8 for CCI-S and n = 6 for CCI-S + WP at 72 hours and n = 6 for sham, n = 7 for CCI-S and n = 6 for CCI-S + WP at 1 week and n = 6 for sham, n = 7 for CCI-S and n = 7 for CCI-S + WP at 16 weeks)

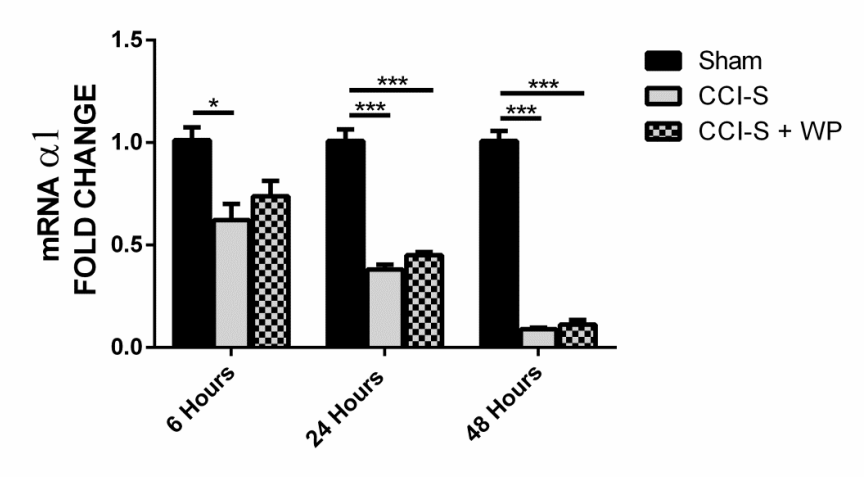

Figure 16. α1 expression is decreased in both CCI-S and CCI-S + WP injured mice 6 hours, 24 hours, and 48 hours after CCI. mRNA levels of α1 were quantified using RT-PCR analysis and represented as histograms showing the fold change of α1 6 hours, 24 hours and 48 hours after CCI in CCI-S, CCI-S + WP and sham injured controls. α1 mRNA levels were normalized to B2M mRNA levels in the same samples and expressed as a fold change compared to sham injured controls (defined as 1). * P < 0.05, ** P < 0.01, P < 0.001 (n = 7 for sham, n = 7 for CCI-S and n = 7 for CCI-S + WP at 6 hours and n = 7 for sham, n = 7 for CCI-S and n = 7 for CCI-S + WP at 24 hours and n = 7 for sham, n = 7 for CCI-S and n = 7 for CCI-S + WP at 48 hours).

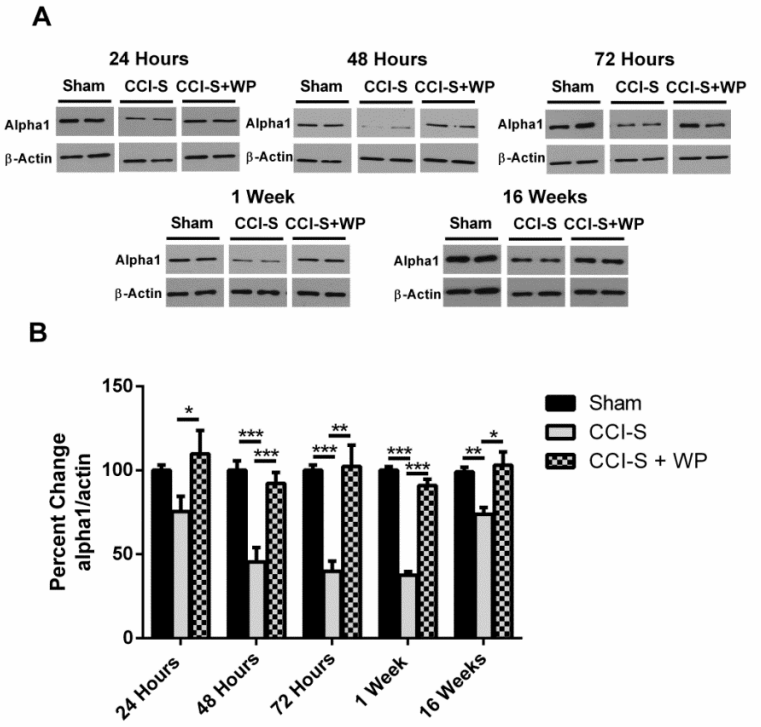

Figure 17. Temporal profile of GABAA α1 subunit receptor after CCI-S with rescues of GABAA α1 subunit receptor with administration of WP1066. (A) Representative western blots from whole hippocampus of mice 24 hours, 48 hours, 72 hours, 1 week and 16 weeks after CCI probed with anti-GABAAR α1 and β-actin antibodies. (B) Quantification of α1 blots showed a significant decrease at 48 hours through 16 weeks after CCI-S injury relative to CCI-S + WP and sham injured controls. α1 levels were normalized to β-actin levels and expressed as a percent change compared to sham injured controls. * P < 0.05, ** P < 0.01, P < 0.001 (n = 7 for sham, n = 7 for CCI-S and n = 6 for CCI-S + WP at 24 hours and n = 7 for sham, n = 7 for CCI-S and n = for CCI-S + WP at 48 hours and n = 6 for sham, n = 6 for CCI-S and n = 6 for CCI-S + WP at 72 hours and n = 6 for sham, n = 7 for CCI-S and n = 7 for CCI-S + WP at 1 week and n = 10 for sham, n = 10 for CCI-S and n = 12 for CCI-S + WP at 16 weeks).

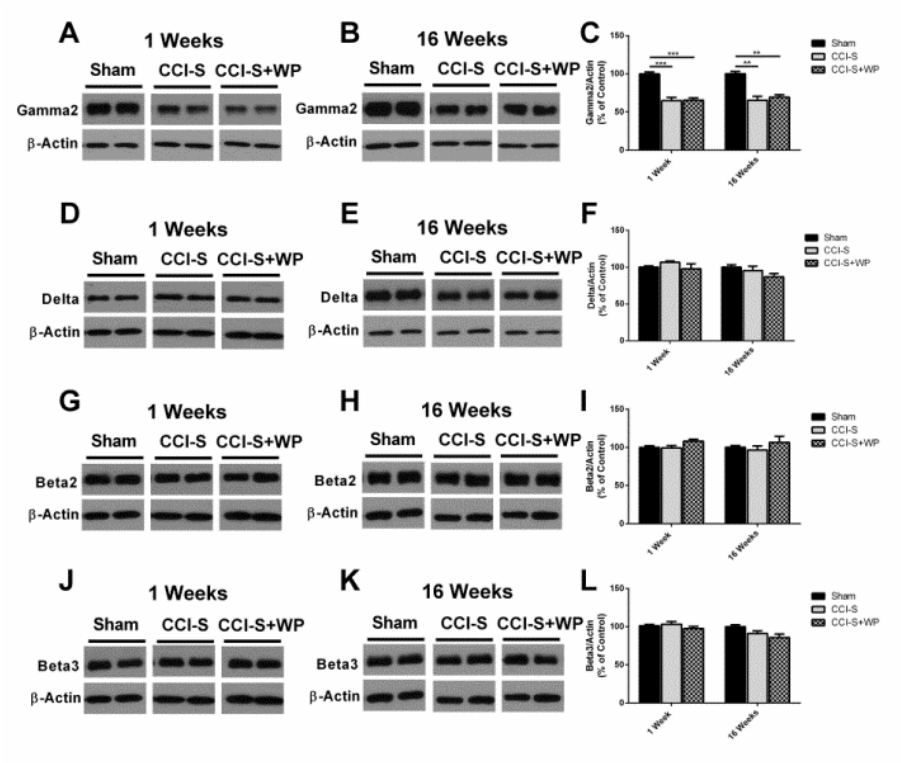

Figure 18. GABAA receptor subunit γ2 is decreased while δ, β3 and β3 are unchanged 1 and 16 weeks post CCI-S and administration of WP1066 does not affect these GABAAR subunit levels. (A) Representative western blots from whole hippocampus of mice 1 week after CCI probed with anti-GABAAR γ2 and β-actin antibodies. (B) Representative western blots from whole hippocampus of mice 16 weeks after CCI probed with anti-GABAAR γ2 and β-actin antibodies. (C) Quantification of γ2 blots showed that the γ2 subunit was significantly decreased 1 and 16 weeks after CCI-S and CCI-S+WP relative to sham injured controls. (D) Representative western blots from whole hippocampus of mice 1 week after CCI probed with anti-GABAAR δ and β-actin antibodies. (E) Representative western blots from whole hippocampus of mice 16 weeks after CCI probed with anti-GABAAR δ and β-actin antibodies. (F) Quantification of δ blots showed that the δ subunit was unchanged 1 and 16 weeks after CCI-S and CCI-S+WP relative to sham injured controls. (G) Representative western blots from whole hippocampus of mice 1 week after CCI probed with anti-GABAAR β2 and β-actin antibodies. (H) Representative western blots from whole hippocampus of mice 16 weeks after CCI probed with anti-GABAAR β2 and β-actin antibodies. (I) Quantification of β2 blots shows that the β2 subunit was unchanged 1 and 16 weeks after CCI-S and CCI-S+WP relative to sham injured controls. (J) Representative western blots from whole hippocampus of mice 1 week after CCI probed with anti-GABAAR β3 and β-actin antibodies. (K) Representative western blots from whole hippocampus of mice 16 weeks after CCI probed with anti-GABAAR β3 and β-actin antibodies. (L) Quantification of β3 blots showed that the β3 subunit was unchanged 1 and 16 weeks after CCI-S and CCI-S+WP relative to sham injured controls. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ (n = 6 for sham, n = 7 for CCI-S and n = 7 for CCI-S+WP at 1 week and n = 10 for sham, n = 10 for CCI-S and n = 12 for CCI-S+WP at 16 weeks).

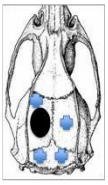

Figure 19: Representative image of the mouse skull with electrode implants and craniotomy. The black circle represents the area of skull which was removed so that the CCI injury could be implemented. The crosses represent the placement of the electrodes (2 recording, 1 ground and reference).

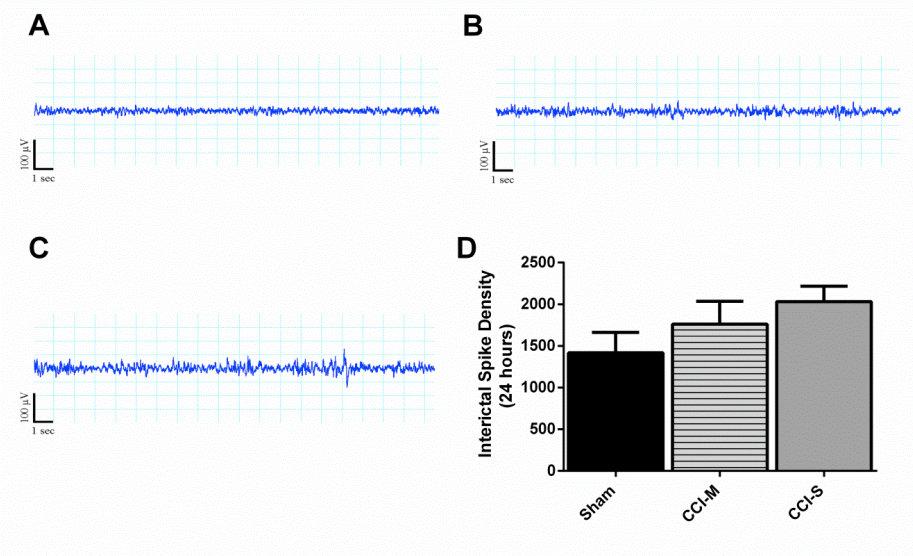

Figure 20. Interictal spiking density is unchanged between CCI-S, CCI-M and sham injured mice 11 weeks post injury. (A) Representative EEG trace of a sham injured mouse 11 weeks post injury. (B) Representative EEG trace of a CCI-M injured mouse11 weeks post injury. (C) Representative EEG trace of a CCI-S mouse injured mouse11 weeks post injury. (D) Quantitation of interictal spiking density showed no differences between sham injured controls, CCI-M injured mice and CCI-S injured mice. n = 5 for sham, n = 6 for CCI-M, n = 13 for CCI-S.

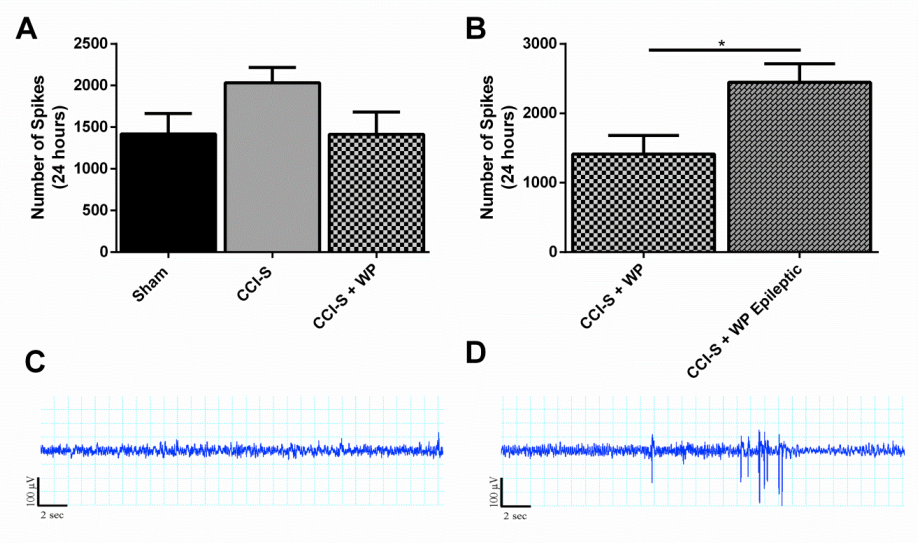

Figure 21. Effects of WP1066 treatment on interictal spiking density.
(A) Quantitation of interictal spiking density showed no differences between sham injured controls, CCI-S injured mice and CCI-S+WP injured mice. (B) Quantitation of interictal spiking showed a significant increase in spiking in CCI-S+WP epileptic mice compared to CCI-S+WP mice that did not develop epilepsy. (C) Representative EEG trace of a CCI-S+WP mouse which did not develop epilepsy. (D) Representative EEG trace of a CCI-S+WP mouse which did not develop epilepsy. * $P < 0.05$, n = 5 for sham, n = 13 for CCI-S, n = 5 for CCI-S + WP and n = 6 for CCI-S + WP epileptic.

Table 2. Comparison of epilepsy development between all groups.
No statistically significant differences in PTE development were found between any of the groups. The only group that had epileptic animals was the CCI-S injury group.

	Sham	Sham + WP	CCI-M	CCI-S	CCI-S + WP
Number of Epileptic Mice	0	0	0	2	4
Total Number of Mice Recorded	5	4	10	16	12
Percentage of Mice that Became Epileptic	**0%**	**0%**	**0%**	**13%**	**33%**

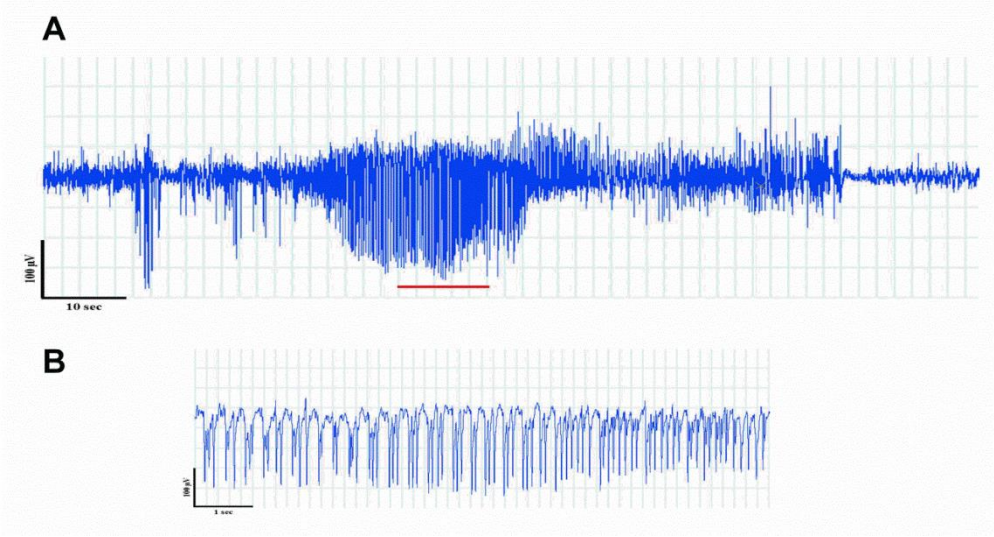

Figure 22. Representative spontaneous seizure from a CCI-S injured mouse. (A) Representative spontaneous seizure that was recorded 10 weeks after CCI injury. This stage 5 behavioral seizure lasted 83 seconds. (B) Zoomed in view of EEG trace showing ictal spiking (red line in figure 20A indicates the 10 seconds that is magnified in 20B).

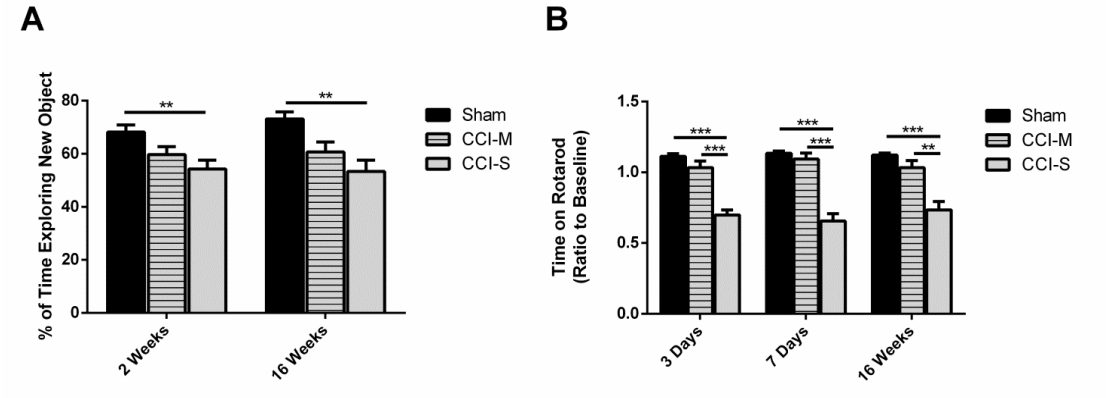

Figure 23. Memory and vestibular motor performance is significantly reduced after CCI-S. (A) Quantification of average time spent exploring the new object shows that the CCI-S injured mice performed statistically worse than the sham injured controls or CCI-M injured mice. (B) Quantification of average time spent on apparatus showed that the CCI-S injured mice performed significantly worse than the CCI-M injured mice or sham injured controls. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ (for A n = 19 for sham, n = 18 for CCI-S and n = 12 for CCI-M at 2 weeks and n = 11 for sham, n = 12 for CCI-S and n = 10 for CCI-M at 16 weeks) (for B n = 20 for sham, n = 28 for CCI-S and n = 19 for CCI-M at 3 days and n = 20 for sham, n = 28 for CCI-S and n = 19 for CCI-M at 7 days and n = 12 for sham, n = 13 for CCI-S and n = 9 for CCI-M at 16 weeks).

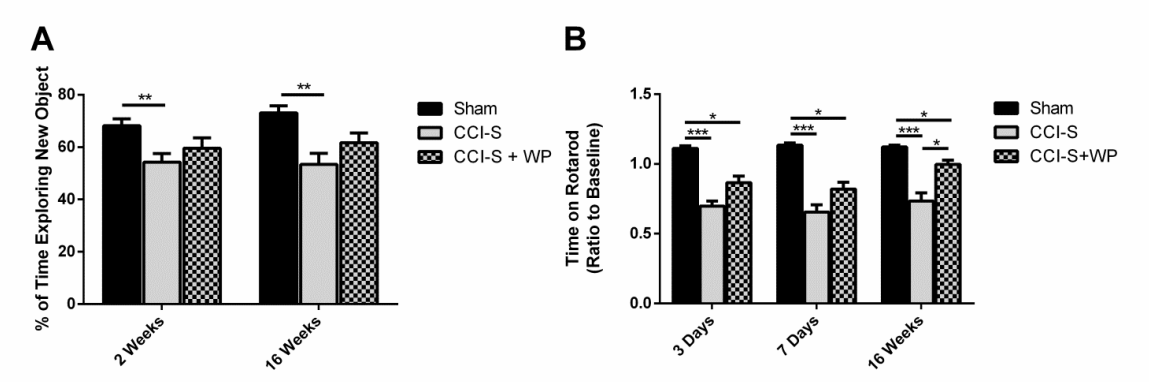

Figure 24. Memory performance is not significantly changed while vestibular motor performance is partially rescued with administration of WP1066. (A) Quantification of average time spent exploring the new object shows that the CCI-S + WP injured mice performed statistically no different than the sham injured controls or CCI-S injured mice. (B) Quantification of average time spent on apparatus shows that the CCI-S injured mice performed significantly worse than the CCI-S + WP injured mice and sham injured controls. * $P < 0.05$, ** $P < 0.01$, $P < 0.001$ (for A n = 19 for sham, n = 18 for CCI-S and n = 14 for CCI-S + WP at 2 weeks and n = 11 for sham, n = 12 for CCI-S and n = 11 for CCI-S + WP at 16 weeks) (for B n = 20 for sham, n = 28 for CCI-S and n = 29 for CCI-S + WP at 3 days and n = 20 for sham, n = 28 for CCI-S and n = 28 for CCI-S + WP at 7 days and n = 12 for sham, n = 13 for CCI-S and n = 12 for CCI-S + WP at 16 weeks).

Appendix #1

Hall, E.D., Smith, B.N., Brooks-Kayal, A., and Soltesz, I. (2014) When ski helmets aren't enough: emerging therapies for TBI and post-traumatic epilepsy. *Winter Conf. Brain Res.*

Traumatic brain injury (TBI) is a major unmet medical need with an annual US incidence >1.5 million persons and a high incidence of lasting neurological deficits and sequelae including posttraumatic seizures (PTS) and posttraumatic epilepsy (PTE). TBI is highly relevant to alpine skiing and snowboarding injuries of which 17.6% are due to TBI. While use of ski helmets has cut down the incidence by as much as 60%, they do not eliminate the risk of TBI and PTE. At present, there are no FDA-approved neuroprotective treatments that improve post-TBI neurological recovery or that prevent PTS and PTE development in contrast to the increasing armamentarium of seizure suppressing compounds (aka anti-seizure drugs), which are not disease-modifying and can have negative effects on cognitive and sensorimotor recovery in TBI patients. This panel will discuss three newer approaches to the treatment of TBI that should attenuate PTE. Following a brief introduction to the epidemiology of TBI, PTS and PTE by organizer Ed Hall, Bret Smith (U. Kentucky) will present elegant studies on the pathophysiology of PTE development in mice subjected to controlled cortical impact TBI that leads to reorganization of circuits in the cortex and hippocampus such that $GABA_A$ receptor (R)-mediated inhibition is decreased while recurrent excitatory circuits are increased. Amy Brooks-Kayal (U. Colorado) will then discuss her recent work on the involvement of the JAK/STAT pathway in the TBI-induced down-regulation of $GABA_A$ R-dependent synaptic inhibition and the utility of JAK/STAT inhibitors for prevention of PTE development. Next, Ivan Soltesz (U. California-Irvine) will discuss the therapeutic potential of optogenetics for treatment of PTE. Finally, Ed Hall (U. Kentucky) will discuss the role of free radical-induced lipid peroxidation in post-traumatic brain damage including PTE development and protective efficacy of newer brain-penetrable antioxidant compounds.